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The viruses and antiviral responses of an invasive fruit pest, *Drosophila suzukii*

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Declaration

I declare that this thesis was composed by myself and that all the work described within is my own. Chapters 2, 3 & 4 are either published or in preparation for submission under the name of multiple authors. I have therefore, used the simple past tense, 'we', to describe the work carried out herein. This work has not been submitted for any other degree of professional qualification except as specified.

Nathan Charles Medd 2/09/18

Table of Content

Contents

Abstract

Drosophila suzukii (Matsamura) is an invasive dipteran pest of soft fruit crops. Native to Japan and SE Asia it was first detected in the Mediterranean growing regions of Europe and the western states of the USA in 2008. Since then it has been expanding its range across both continents causing huge economic damage to the horticultural industries there. Current control measures are heavily dependent on broad spectrum insecticides and labour intensive cultural control. Therefore, there is a large incentive to investigate alternative, more environmentally benign, control methods such as biological control or biopesticides.

The viruses of *D. suzukii* offer a potential source of pathogens suitable for the development of such a biopesticide. Chapter 2 explores the diversity of viruses found naturally associating with *D. suzukii* in both its native and naturalised ranges. In it, I describe 18 new RNA viruses belonging to a variety of virus clades. Although none of these viruses belong to those clades traditionally used as biological control agents, we suggest further work for the development of a viral control agent based on our data.

Not only are the viruses of *D. suzukii* of direct applied interest to the horticultural industry, they also offer a powerful model system for the study of virus host dynamics in the wild. The ecosystems recently invaded by this pest contain many other species of *Drosophila* which harbour their own raft of viral pathogens. In chapter 3 I explore the extent to which these viruses are shared between species and how virus prevalence changes over time. Understanding the patterns of virus 'host-shifts' after

host range change could help us better predict the success of particular biological invasion events and further informs our understanding of emerging viral diseases in both humans and livestock.

The ability of a virus to shift host ultimately comes down to its ability to overcome its host's immune system. In chapter 4 I investigate the comparative genome-wide transcriptomal immune responses of *D. suzukii* and its congener *D. melanogaster* after treatment with two highly divergent viruses. The relative responses of these flies was shown to be highly dissimilar as was the response of males and females of the same species. Few model species allow comparative expression studies of this depth granting us unprecedented insights into the evolution of insect innate immune systems.

Lay Summary

In a globalised world we are familiar with the interconnectivity of countries across the planet. Borders for trade and communication are increasingly reduced as technology and travel become more and more frictionless. This connectivity spills over into the animal kingdom. We are accidentally, sometimes even intentionally, introducing animals and plants from one side of the globe to another. Organisms once incapable of long distance migration can now stow away along international trade routes, exploiting new habitats inaccessible before the activities of modern man. This can be a big problem for those invaded ecosystems, having potentially disastrous effects on ecosystem function, ecosystem services, and the productiveness of agricultural systems. One relatively understudied factor determining whether an alien species succeeds in its invasion is the relationship it has with its diseases as it invades. There is potential for the invasive species to experience new pathogens, leaving its familiar ones behind. Better understanding the relationship of invasive species with their pathogens could help us predict biological invasions and could even give us the weapons to fight the invasion. 'Biological control' is the use of an organism's natural enemies to control its population and it's a technique that can be very effective against invasive pests.

In this study we have explored the diversity of viruses infecting an invasive fruit fly, *Drosophila suzukii*, which is currently sweeping across the globe causing massive damage to fruit crops as it goes. In the second chapter of this work I describe 18 new viruses, discovered in this pest, some that could be potentially useful as biological control agents. I then, in the second chapter, describe the ecology and host range of some of these viruses, asking: what viruses are being shared between similar fly species? I then delve into how this pest fights viruses, examining its immune system in comparison to a closely related but benign fruit fly.

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1. INTRODUCTION

Drosophila suzukii (Matsummura) is an invasive dipteran pest of soft fruits. Its recent invasion of the fruit growing regions of North America and Europe and the damage it has caused there have driven interest in finding new control solutions. Conventional chemical control methods have many drawbacks and are difficult to implement, consequently the development of an integrated pest management (IPM) compatible biopesticide would be extremely beneficial for growers, consumers, and pest management professionals alike.

This study seeks to characterise the viral diversity of *D. suzukii* with the aim of identifying a pathogen suitable for the control of this pest in UK fruit crops. To do this we first used a metatransciptomic approach to identify viral sequences from wild *D. suzukii*. We then investigated the patterns of virus infection in several species of wild British *Drosophila*, giving us a picture of virus ecology and host specificity. Finally, to assess the susceptibility of *D. suzukii* to viral infection we conducted a comparative analysis of immune system gene expression between *D. suzukii* and the closely related *D. melanogaster*.

DROSOPHILA SUZUKII

Belonging to the paraphyletic subgenus Sophophora and the *melanogaster* species group, *D. suzukii* is phylogenetically close to the famous lab model *Drosophila melanogaster* (Lewis et al., 2005, Kopp, 2006). Some striking morphological characters do, however, allow *D. suzukii* to be distinguished from its well-studied relative. Amongst these the presence of dark wing spots in the male (giving rise to the common species name 'Spotted Wing *Drosophila*') and a heavily sclerotized

ovipositor bearing tooth-like bristles in the female are most prominent. It is this uniquely well-developed ovipositor that is considered to be the evolutionary innovation that allows *D. suzukii* to oviposit under the skin of ripening fruit still on the tree. This feature, although ostensibly shared by a small number of other *Drosophila* species, nowhere else results in the ability to pierce such a range of fruit skins (Atallah et al., 2014a). Once laid, the eggs of *D. suzukii* develop through three larval instars inside the fruit, feeding on the mesocarp. Complete development, from egg to adult, takes approximately 8 to 10 days at 25 °C, and from 21 to 25 days at 15 °C according to early life history studies (Kanzawa, 1935, Kanzawa, 1939). Further information on oviposition behaviour is provided by (Mitsui et al., 2006). Here, the authors observed the distribution of oviposition in wild, native *D. suzukii*, noting that egg clutches were randomly distributed and predominantly consisted of single egg clutches. This compared to two other Asian *Drosophila* species in the study, *D. lutescens* and *D. rufa* which both distributed multiple egg clutches in a significantly more clustered pattern. Wild invasive populations have also been studied in respect to host range and overwintering in more recent studies on the species (Walsh et al., 2011) . Walsh et. al. (2011) tested the ability of all life stages of *D. suzukii* to overwinter in typical Oregon winter conditions, finding that adults were the most robust overwintering stage with 39% of adults surviving 60 days of simulated winter conditions.

The monitoring scheme in the UK has reported the number of *D. suzukii* adults, caught in bait traps, to peak at some point between September and November depending on weather conditions. As British records of *D. suzukii* only date back three growing seasons, data on the phenology of the organism is still limited.

A very broad range of host plants makes *D. suzukii* an especially difficult pest to control. *D. suzukii* is known to oviposit in a wide variety of commercial and wild soft skinned fruit (Walsh et al., 2011, Cini et al., 2012, Mitsui et al., 2010). This allows populations to reside in wild refuges and may facilitate the reinvasion of crops after periods of intense spraying, fruit unavailability or cold weather. Further work on the small to medium scale population dynamics and ecology of this species are desperately needed to aid control.

PATTERN OF INVASION

First described in Japan in 1916 (Matsumura, 1931), *D. suzukii* was reported to be widely distributed in Japan shortly after (Kanzawa, 1939). This species was then subsequently recorded across Asia during the last century: China (Peng, 1937), North and South Korea (Kang and Moon, 1968, Nagayama and Okamoto, 1940), India (Parshad and Duggal, 1965), Thailand (Okada, 1976), Burma (Toda, 1991), Eastern Russia (Sidorenko, 1992) and Pakistan (Amin ud Din et al., 2005). Recent studies, examining the genetic diversity within and between populations of *D. suzukii* populations from around the world, found Japanese populations had the largest number of unique haplotypes, supporting the theory that Japan falls within this species native range (Adrion et al., 2014, Carvajal and Markow, 2010). The first records of this pest from outside Asia came from Hawaii in the 1980's (Kaneshiro, 1983). Several, more recent, records of *D. suzukii* in Hawaii have been published (Beardsley et al., 1999, O'Grady et al., 2002), however, no crop damage is reported from these islands, despite the well-developed fruit growing industry there. This apparent lack of damage is, therefore, presumably due to some suppression of *D.*

suzukii populations in Hawaii, by natural enemies of some sort. Since its detection in Spain during 2008 (Calabria et al., 2012), *D. suzukii* has spread northwards through continental Europe (Vogt et al., 2012, Seljak, 2011, Baroffio and Fischer, 2011) and was reported for the first time in the UK in 2012 (Harris and Shaw, 2014). Further records of detection continue to be published from across Northern and Central Europe (Lavrinienko et al., 2017, Manduric, 2017, Kiss et al., 2016, Piotrowski et al., 2016), with the northerly most detection being in Scandia, Sweden (Manduric, 2017). A recent study found *D. suzukii* to be one of the four most abundant drosophilid species in the growing regions Apulia, Italy ((Antonacci et al., 2017).

Parallel to its spread across the Western Palearctic region, *D. suzukii* has simultaneously invaded the Neartic and Neo-tropical ecozones. The first detection of this species was logged in California (Bolda, 2008), with records soon following from across the western USA (Bolda et al., 2010, Goodhue et al., 2011). The pest was also detected on the eastern seaboard shortly after (Price et al., 2009) and is confirmed to now be breeding in wild fruit in the North Eastern states (Maier, 2012). The patterns of genetic diversity across the USA suggest a scenario in which colonisation has been passively mediated (anthropogenically or by wind) rather than through active dispersal by the species (Adrion et al., 2014). The pest has now been recorded from Canada, from British Columbia in the west (Bolda et al., 2010) to Dunham, Quebec in the east. Spreading southwards, *D. suzukii* has been recorded across Brazil (Deprá et al., 2014) with current records ranging as far south as south Argentina (Lue et al., 2017).

ECONOMIC DAMAGE

Drosophila suzukii has the potential to cause severe damage to commercial soft fruit crops. During oviposition the female fly punctures the skin (exocarp) of the ripening fruit with her oviscapt. Even if no subsequent larval feeding takes place this wound allows fungi to begin degrading the fruit, rendering it unsalable. In cases where larval feeding occurs in the flesh (mesocarp), the fruit often collapses entirely also leaving that fruit unmarketable. Where *D. suzukii* has established, substantial (up to 80%) crop loss has been reported on a variety of soft skinned fruit crops (Walsh et al., 2011).

The impact of this pest on the European horticultural industry has already been substantial with *D. suzukii* infestations resulting in losses of over €8 million in fruit crops in Northern Italy in 2010 and 2011 and more than €1.5 million for French strawberries in 2011 (FERA, 2015). The European and Mediterranean Plant Protection Organisation (EPPO) in a recent 'Pest Risk Analysis' deemed this organism to be a potential threat to crops in its region. Potential damage is described as "massive" and the regions ability to control it as "with much difficulty" (EPPO, 2010).

In the Pacific fruit growing regions of the USA, the estimated damage due to *D. suzukii* has been calculated at over €400 million/year (Bolda et al., 2010). In Californian raspberries specifically, the damage caused by *D. suzukii* between 2009 and 2014 has been calculated at $US 39.8 million in revenue losses, equivalent to 2.19% of realized revenues (Farnsworth et al., 2017).

CONTROL

In light of the rapid spread of *D. suzukii* and potentially serious economic damage it can cause, a huge imperative lays on finding an effective control programme for this

pest. Mitigation of the worst economic damage is possible through proper management programs. Fava *et al.* (2017) calculated that economic loss from D. suzukii infestations can be mitigated by 3.24% with the introduction of IPM strategies during low pressure periods, with an upgraded IPM programme (including netting) being increasingly more profitable during periods of high pest pressure.

The challenge for crop protection scientist is intensified by the biology of this particular organism: a short generation time, wide host range and cryptic feeding stages in close-to-harvest fruit combine to hinder conventional control. Furthermore, control techniques are often sought in crops with existing management programmes designed to control a range of pests whilst limiting chemical input, known as integrated pest management or IPM.

Integrated pest management programmes attempt to introduce alternatives to chemical pesticides: reducing the environmental impacts of pest control, managing resistance to pesticides, improving grower safety and reducing chemical residues in produce. This is achieved through the use of non-chemical control methods. These include: biological control; the introduction or augmentation of the pest's predators, parasitoids or pathogens, cultural control; preventative techniques such as plant variety selection or crop hygiene that pre-emptively reduce the susceptibility of a crop to pest attack and mechanical control; techniques that involve barriers, i.e. netting, or the physical removal of pests. Alongside non-chemical methods, the responsible use of synthetic pesticides, often those with a low environmental impact or high target specificity, also forms a part of most IPM programmes. The practice of

IPM is underpinned by the concepts of monitoring and economic injury thresholds, shifting the emphasis from complete eradication of pests to population maintenance and damage mitigation. IPM techniques have an increasing uptake in orchard and protected fruit crops, becoming particularly commonplace in Europe during the last decade. For a detailed review see Lefebvre et al. (2015). The introduction of a new pest, with no IPM compatible products available for its control, can disrupt or even destroy well established integrated programmes as the emergency use of broad-spectrum pesticides kill biological control agents and lead to secondary pest resurgence.

Many current control strategies for *D. suzukii* include an element of high volume, short persistence, pesticide sprays. High volume pesticide applications are undesirable for most parties involved in fruit production, firstly because of potential pesticide residue issues: As *D. suzukii* oviposits close to the time of harvest, targeted applications may cause unwanted residue on fruit at point of sale. Most fruit buyers, including supermarkets, and regulators have extremely low tolerances for pesticide residue, due largely to customer demand for pesticide free produce (Collins et al., 1993). A reduction in residues has been key driver in the development of IPM programmes in soft fruit (Cross and Berrie, 2006). Secondly the application of broad spectrum insecticides, as currently advocated for control of *D. suzukii* (Bruck et al., 2011), can have local environmental consequences that, not only effect wild ecosystems but also harm potentially useful biodiversity with the cropping system (reviewed in: Desneux et al., 2007, Biondi et al., 2012, Fountain and Medd, 2015, Crowder and Jabbour, 2014). Thirdly, high volume spray programmes run the risk of

driving the rapid development of insecticide resistance in target and non-target pests. This has been the case for a number of invasive crop pests where pesticide resistance has developed within non-native populations: The tomato pinworm, *Tuta absoluta* (Campos et al., 2014); the Colorado potato beetle, Leptinotarsa decemlineata (Sukhoruchenko and Dolzhenko, 2008, Sharif et al., 2007, Zamojska et al., 2011, Pourmirza, 2005, Stanković et al., 2004); the Asian citrus psyllid, *Diaphorina citri* (Tiwari et al., 2011); and the Q type tobacco whitefly, *Bemisia tabci* (Luo et al., 2010), to name just a few notable examples. Insecticide resistance is a well-studied area of evolutionary biology and consequently a good understanding of the genetic mechanisms behind resistance has been achieved, especially in *Drosophila*, which serves as a useful lab model for the study of insecticide resistance (Morton, 1993).

IPM compatible solutions for *D. suzukii* infestation are, however, emerging. Cultural control, in the form of crop hygiene, currently plays a large part in the control of *D. suzukii*. Collecting, neutralising and disposing of fruit waste correctly, although time consuming, has proven effective and is an important part of control recommendations disseminated to growers (ADHB, 2015). Increasing the overall number of harvests per week, shortening the amount of time that ripe fruit spends, vulnerable to attack, on the crop, is also proving a simple but effective measure to control populations of this pest (Cross, *pers comms*). Trapping has also formed a key component of many *D. suzukii* control programs to date. With various trap types and baits commercially available and a range of placement strategies proven to be effective (Lee et al., 2012, Grassi et al., 2014, Lee et al., 2013, Cha et al., 2013, Cha et al., 2015). Trapping is generally environmentally benign and compatible with existing

IPM programmes. Placement of traps does, however, pose a large investment in labour time and expense for growers (Mazzi et al., 2017, Del Fava et al., 2017).

Studies into the biological control of *D. suzukii* using invertebrate natural enemies have given mixed results. Several studies have shown resistance in *D .suzukii* to attack by European parasitoid wasps (Chabert et al., 2012, Kacsoh and Schlenke, 2012, Poyet et al., 2013), whilst others report the spontaneous parasitism of *D. suzukii* in the field (Gabarra et al., 2014, Stacconi et al., 2013, Miller et al., 2015) and successful parasitism in controlled laboratory settings (Rossi Stacconi et al., 2015). Kacsoh and Schlenke (2012) and Poyet et al. (2013) report an association between resistance in *D. suzukii* to parasitoid attack and high haemocyte load in infected individuals. This correlation between increased haemocyte load and resistance to parasitoids has been noted for a number of other species in the melanogaster species group (Eslin and Prévost, 1998), however, total haemocyte load does not appear to be correlated with ability to encapsulate parasitoids in *D. melanogaster* itself despite a high natural variation in encapsulation ability across different European field collected lines (Gerritsma et al., 2013). *D. suzukii* also appears to increase its resistance to parasitoid attack through 'self-medication', i.e. preferentially laying eggs on substrates containing high levels of atropine, an entomotoxic alkaloid, in the presence of parasitoids (Poyet et al., 2017). A similar behavioural immune response is also seen in *D. melanogaster* (Kacsoh et al., 2013).

Several studies have identified potential predators of *D. suzukii* in the predatory hymenopteran genus *Orius*. These small predatory bugs, or pirate bugs, are currently widely used as inundative biological control agents in covered horticulture. *Orius*

leavegatus has been recovered from the field in *D. suzukii* vulnerable crops (Gabarra et al., 2014, Arnó et al., 2012) and proved an efficacious predator of *D. suzukii* eggs in lab condition strawberry fruits (Gabarra et al., 2014). *O. leavegatus* has also been shown to feed on *D. suzukii* adults under lab conditions (Cuthbertson et al., 2014b), however, neither *O. maiusculus*, *O. insidiosus* nor *O. leavegatus* proved particularly voracious in other lab conditions (Malagnini et al., 2014, Woltz et al., 2015) and their role in population suppression in the field remains questionable. Other generalist predators, earwigs for example may have a marginal role in supressing *D. suzukii* by consuming exposed larvae or pupae (Gabarra et al., 2014) but again these cannot be relied upon in isolation.

Another key branch of many IPM programmes is the use of microbial biopesticides. The susceptibility of *D. suzukii* to a number of microbial biological control agents has been tested. Several species of entomopathogenic fungi significantly reduce *D. suzukii* survival in laboratory assays: *Metarhizium anisopliae* (Woltz et al., 2015), *M. brunneum* (Cossentine et al., 2016, Fernández-Bravo, 2014), *Beauveria bassiana* (Cossentine et al., 2016, Cuthbertson et al., 2014a, Gargani et al., 2013, Cuthbertson and Audsley, 2016), *Lecanicillium muscarium* (Cuthbertson et al., 2014a), *Lecanicillium lecanii* (Cossentine et al., 2016) and *Isaria fumosorosea* (Cuthbertson and Audsley, 2016, Cossentine et al., 2016, Naranjo-Lázaro et al., 2014). Primary bioassays are, obviously a key first step to implementing any control measure, however, there is a need for more field scale data on the effectiveness of currently available microbial pesticides. Delivery methods, critical for success in ensuring the

necessary spore-to-cuticle contact, along with a whole host of other variables must be tested before solid advice can be given to growers.

MICROBIAL PESTICIDES

Microbial based biopesticides have a long history in crop protection with experiments involving biological controls for insect pests dating as far back as 1835, when Agostine Bassi demonstrated that white-muscadine fungus (*Beauveria bassiana*) was the causative agent of an infectious disease in silkworm (BPIA, 2015). The most widely used microbial biopesticides are those containing the spores and insecticidal crystalline proteins of *Bacillus thuringiensis* (Bt) with products such as DiPel® and Thuricide® gaining widespread acceptance and use in the horticultural industry. The inclusion of Bt *Cry* genes into transgenic crop genomes, resulting in the expression of the insecticidal proteins by the plant, further developed the use of this microorganism in agricultural crop protection (Vaeck et al., 1987). During the 1990's Bt products made up 90% of the global biopesticide market, though it's market share has now decreased (55% in 2012) as Bt spray use is replaced by Bt transgenic crops and the number and volume of other biopesticide products increase (de Maagd, 2015). Many other microbial products now grace the crop protection market and although they command a small proportion of the global pesticide market (Thakore, 2006) they are increasing in popularity as product efficacy, reliability, production technologies improve, for review see Glare et al. (2012).

The viruses of *D. suzukii* offer an interesting potential source for a microbial biological control agent. Similarly to microbial biological control agents: viruses potentially represent an environmentally benign control agent with high host specificity and low

environmental persistence (Hunter-Fujita et al., 1998b), making them eminently suitable for inclusion into existing IPM programs. Although some hurdles exist in the commercialisation of insect viruses as control agents (Carter, 1984), the improvement of culturing technologies and the rationalisation of restrictive regulations may, in time, alleviate some of the current difficulties (Sun and Peng, 2007).

CHARACTERISTICS OF VIRAL BIOLOGICAL CONTROL AGENTS

Entomopathogenic viruses are represented in many of the known virus families with some families of virus are known to occur solely in arthropods (Hunter-Fujita et al., 1998). Commercial success as a plant protection products has, however, been achieved only by a small selection of viruses. The two most notable both belonging to the family Baculoviridae.

The family Baculoviridae consists of 600 described species in two genera: the Nuclear polyhedrosis viruses (NPV's) and the Granulosis viruses (GV's)(van Regenmortel et al., 2000). Only known to naturally infect arthropods, these viruses have been studied not only for their suitability as control agents but for their application in molecular biology as expression vectors (Smith et al., 1983, Luckow and Summers, 1988). Different species of baculovirus have been isolated from many different insect orders (Hunter-Fujita et al., 1998b) but their deployment as biopesticides has mainly been against Lepidopteran pests (for review see Moscardi (1999)). Baculoviruses are enveloped and have a double stranded DNA genome of 80 to 200kb in length. Extracellular virions can be found in two forms: budded virions (BV's) which are formed during cell-to-cell transmission, or packaged in an occlusion body (OB) during

host-to-host transmission (Granados, 1980). A feature almost unique to insect viruses, an OB is a proteinaceous, mainly polyhedrin, lattice that protects virions from the environment. Occlusion bodies vary in size from between 0.5 to >20µm in diameter and are often visible under a light microscope. Two other virus families contain occulated insect viruses: the dsRNA Reoviridae subfamily Spinareovirinae (Cytoplasmic polyhedrosis viruses, CPV) and the Poxviridae, specifically the subfamily Entomopoxvirinae. Although many other, non-occluded, virus families infect insects, an occluded virus would be an ideal candidate for the development of a biological pesticide due to its environmental resilience.

Other viruses endorsed and tested for the control of insect pests belong to two other virus families: the Nudiviridae and the Parvoviridae. Oryctes nudivirus is a non-occluded dsDNA virus that was first described as *Rhabdionvirus oryctes* (Huger 1966). It was later defined as Oryctes virus and placed in a subgroup of the Baculoviridae by the International Committee on Taxonomy of Viruses (ICTV) before being incorporated into the Nudiviridae and designated as Oryctes rhinoceros nudivirus (OrNV) (Wang et al. 2007). This virus was introduced into Samoa in 1963, and later to other Pacific Ocean islands, to control the Coleopteran pest of cultivated Palms: *Orytes rhinoceros.* The virus is lethal to larvae and causes feeding cessation in adults and consequently led to huge declines in pest population over the course of 1-3 years. A reduction in crop damage accompanied the reduction in population. Reapplication in areas of pest resurgence has proved effective, however, after 40 years a breakdown in control in certain locations is being reported by researchers (Jackson, 2009, Huger, 2005). The virus has been studied extensively in India where successful

control of O. rhinoceros has also been achieved (Mohan and Pillai, 1993, Gopal et al., 2001). Closely related nudiviruses have recently been discovered in *Drosophila* (Unckless, 2011, Webster et al., 2015). A genus of the virus family Parvoviridae, the densoviruses or densonucleosis viruses (DNV's) are another group of viruses with potential use as viral insecticides. These single stranded DNA viruses were first discovered infecting the greater wax moth *Galleria mellonella* by Meynadier et al. (1964). Since that point they have been subsequently isolated from a range of insect taxa, see Maramorosch (2012). No publications report their isolation from *Drosophila*, however, evidence of their presence has been detected in *Drosophila* transcriptome datasets (Obbard, *pers. comms.*). They have been advocated for the control of Mosquitoes (Carlson et al., 2006, Ledermann et al., 2004) and cockroaches (Jiang et al., 2008) although field studies into their application are yet to be published.

DROSOPHILA VIRUS DIVERSITY

Viruses are a ubiquitous threat to all living organisms. Possibly one of the first parasites (Koonin and Dolja, 2013) they have been applying constant evolutionary pressure to their hosts since the birth of life on this planet. No organism is free from viruses, yet viruses are known from a comparatively few species of medical, economic or conservation importance. This is beginning to change. Modern metatranscriptomic techniques have allowed a surge in the numbers of insect viruses described (Shi et al., 2016) and the genus Drosophila is no exception. Studies by Webster *et al.* (2016, 2015) reported over 50 new viruses from the genus. Prior to these survey efforts only 11 viruses were known in *D. melanogaster* (Brun and Plus, 1980, Huszar and Imler, 2008) with only five of these isolated, sequenced and available for experimental study:

Drosophila melanogaster sigma virus (DmelSV), *Drosophila* C virus (DCV), *Drosophila* A virus (DAV), *Drosophila* Nora Virus and *Drosophila* X virus (DXV).

Sigma virus (DmelSV) was the first virus to be discovered in *Drosophila* (l'Héritier and Teissier, 1937). It was discovered by chance due to an unusual symptom of CO_2 sensitivity in infected flies and was later found to be transmitted vertically through eggs and sperm but also to be transmissible through injection, identifying a virus as the causal agent (L'Heritier, 1948). Further examination of the virus lead to its classification into the family rhabdoviridae (Teninges, 1968, Berkalof et al., 1965, Teninges et al., 1993). DmelSV is not the only sigma virus to infect *Drosophila*: *D. affinis*, *D. obscura*, *D. tristis*, *D. immigrans* and *D. ananassae* were all found to be infected with sigma viruses by screening for CO_2 sensitivity (Longdon et al., 2009, Longdon et al., 2011b).

Drosophila C virus (DCV) was first isolated in a French strain of *D. melanogaster* (Jousset et al., 1972) and has since become one of the most well studied viruses of *Drosophila* (Huszar and Imler, 2008, Jousset et al., 1977). Closely related to another well studied insect virus, the Cricket Paralysis Virus (CpV), DCV belongs to the family Dicistroviridae. DCV is lethal to *D. melanogaster*, infecting the muscles around the fly's crop, foregut, casing acute cytopathology and intestinal obstruction in adult flies (Chtarbanova et al., 2014).

Two less well studied viruses of *D. melanogaster* that afford mention are DAV and Nora virus. DAV is an unusual RNA virus described initially as a picorna-like virus (Brun and Plus, 1980, Plus et al., 1976) but with a diverse range of biological attributes that

make it difficult to place systematically (Ambrose et al., 2009). It exhibits low pathogenicity in its host (Brun and Plus, 1980) despite interacting with antiviral RNAi pathways and has a global prevalence of between 5 and 10% (Webster et al., 2015) Also described as a picorna-like virus, *Drosophila* Nora virus is a small non-enveloped RNA virus infecting *D. melanogaster* and the closely related *D. simulans* (Habayeb et al., 2006). This virus is transmitted horizontally and has little effect on the longevity or fecundity of infected flies (Habayeb et al., 2009).

Drosophila X virus (DXV) is a non-enveloped dsRNA virus belonging to the family Birnaviridae. It was first discovered as a contaminant in a study on DSV in cell lines (Dobos et al., 1979). Little is known about the replication cycle of DXV and it has never been found as a natural pathogen of wild *Drosophila*. It has, however, been detected in *Culicoides* sp. (Adams and Bonami, 1991). The exact origin of the original contamination is not known.

Few studies have focused on the diversity of viruses in wild *Drosophila* populations. Recently, however, the development of metagenomic techniques has facilitated a new approach to viral discovery and has expanded our knowledge of insect virus diversity immensely (Liu et al., 2011). (Webster et al., 2015) used next generation sequencing technology to identify more than 20 previously undescribed RNA and DNA viruses associated with *D. melanogaster*. Their survey of over 2000 individual wild flies showed 30% of flies to carry at least one virus and 6% of flies to carry multiple viruses. This study also involved the analysis of publically available RNA-seq datasets to estimate viral prevalence in laboratory stocks.

Less is known about the viruses infecting other species of *Drosophila* in the wild, *D. melanogaster* being by far the best studied. 25 new viruses, discovered through metatranscriptomic surveys were, however, described by Webster et al. (2016) in a number of British *Drosophila* species. Between one and five new viruses were described from pooled samples of the species: *D. tristis*, *D. subsilvestris*, *Scaptodrosophila deflexa*, *D. obscura*, *D. subobscura* and *D. immigrans.*

A study by Unckless (2011) identified a DNA nudivirus infecting wild *Drosophila innubila*. This viruses is closely related to the OrNV discussed above for its use as a biological control agent of coleopteran palm pests. Also closely related to OrNV, a nudivirus of *D. melanogaster* was discovered by Webster et al. (2015). Named Kallithea virus, this virus was found to be relatively common in wild *D. melanogaster* (4.6% prevalence globally) and was shown to be interacting with antiviral immune pathways in its host.

ANTIVIRAL IMMUNITY IN *DROSOPHILA*

Insects rely almost entirely on an innate immune response, as opposed to the familiar, adaptive, immune response found solely in vertebrates. Several of the pathways involved in innate antiviral immune response were first identified in *Drosophila* and have since been proven to be highly conserved amongst the invertebrates and vertebrates alike. In *Drosophila* a range of different pathways are thought to be involved in the innate antiviral response: the Toll pathway, IMD pathway, JAK/Stat pathway, Toll-7 autophagy pathway, transcriptional pausing pathway and the RNA interference pathway, reviewed in (Sabin et al., 2010). All start with pathogen recognition. Pattern recognition receptors (PRRs) recognise conserved components

of different pathogens by what are known as pathogen-associated molecular patterns (PAMPs). These receptors recognize conserved components of different pathogens, including viral glycoproteins and viral genetic material (Takeuchi and Akira, 2010) There are several distinct classes of PRRs, acting as either membrane bound sensors (Toll-like receptors or C-type lectin receptors) or cytoplasmic sensors (Retenoic acid-inducable gene-like receptors or NOD-like receptors) (Akira et al., 2006).

Once binding of PAMPs signals pathogen recognition, signalling pathways are activated resulting in the transcriptional activation of a certain subsets of genes leading to the production of effector molecules that suppress pathogen replication. Two important immune signalling pathways downstream of PRR's in Drosophila, are the Toll and IMD pathways. Both rely on the nuclear factor-κB (NF- κB). The Toll pathway, first discovered in Drosophila, shares similarities with the mammalian Toll-like receptor signalling pathway subsequently described in mammals (Fitzgerald and Chen, 2006, Lemaitre and Hoffman, 2007). The Toll pathway is predominantly associated with antibacterial and antifungal defence in insects but has been found to protect against some viral infections in insects: Toll signalling is induced by and restricts DXV infection in Drosophila and Dengue infection in Aedes mosquitoes (Xi et al., 2008, Ramirez and Dimopoulos, 2010).

The other canonical NF- κB immune pathway downstream of PRRs is the immune deficiency (IMD) pathway. It is often compared to the mammalian Tumor Necrosis Factor Receptor (TNFR) pathway as they share several conserved components

(Myllymäki et al., 2014). Like the Toll pathway, IMD signalling regulates the production of effector molecules, antimicrobial peptides (AMP) that are primarily associated with the suppression of bacterial and fungal infections. Despite this association, the IMD pathway has also been implicated in antiviral defence with isogenic mutants certain IMD pathway components being more sensitive to infection by Cricket Paralysis virus (CrPV)(Costa et al., 2009).

A third pathway implicated in Drosophila antiviral defence is the JAK/STAT signalling pathway. The pathway consists of four main components: the ligands, unpaired (upd1-3), which bind to the receptor domeless (Dome), which signals through the kinase JAK (Hopscotch/Hop), to activate the transcription factor STAT (STAT92E/Marelle) resulting in the production of antimicrobial effectors including the antiviral vir-1(Xu and Cherry, 2014). Vir-1 has a known association with Drosophila antiviral defence: *Drosophila* C virus (DCV) and Flock House virus (FHV) both inducing an up-regulation of the Jak-STAT induced *vir-1* gene (Dostert et al., 2005, Hedges and Johnson, 2008).

Probably the most important pathway in antiviral response is thought to be that of RNA interference (RNAi) pathway (Zambon et al., 2006, Obbard et al., 2009a, Bronkhorst and van Rij, 2014). Three RNAi pathways have been identified in *Drosophila*: the small-interfering (si)RNA pathway, the micro (mi)RNA pathway and the PIWI interacting (pi)RNA pathway (reviewed by Kim et al. (2009)). The siRNA pathway is most often associated with the antiviral response in insects. On uptake of viral dsRNA (Saleh et al., 2009) 'Dicer' proteins in the cytoplasm recognise and bind

to viral dsRNA, cleaving it into siRNA fragments and initiating the pathway (Ding and Voinnet, 2007). These siRNAs are then loaded in to the RNA induced silencing complex (RISC) which guides the slicing enzyme Argonaut to complementary viral RNA sequences which are in turn cleaved preventing viral replication. Recently, an additional element to this response has been reported whereby a form of systemic memory is exhibited (Tassetto et al., 2017, Saleh et al., 2009, Attarzadeh-Yazdi et al., 2009, Poirier et al., 2018), in which haemocytes endogenise fragments of RNA virus as DNA copies, and these endogenous copies form a source of secondary viral siRNAs.

ECOIMMUNOLOGY OF D. SUZUKII

As an invasive species, *D. suzukii* caught in the UK today are potentially is experiencing a different immunological environment to their recent ancestors. Rapid introduction into a new ecosystem can bring with it a reduction in the diversity of natural enemies adapted to prey on or infect the invasive organism, a concept known as the enemy realise hypothesis or ERH, (Keane and Crawley, 2002). Indeed a reduction in the number of compatible enemies, or their effect on the introduced species, has been demonstrated for numerous different invasive organisms in their naturalised ranges (Callaway et al., 2004, Torchin et al., 2001, Wolfe, 2002, Beckstead and Parker, 2003), especially on the leading edge of an invasion where parasites have been found to lag behind their hosts (Phillips et al., 2010). This reduction could in turn impart an ecological advantage to the invasive species, aiding range expansion and establishment, not only by a reduction in extrinsic population control but by providing an evolutionary opportunity to reallocate resources away from costly defences (Blossey and Notzold, 1995). Although seemingly intuitive evidence for the

ERH is incomplete and the true reasons for increased abundance or impact of introduced species may be far more complex (Colautti et al., 2004). It has been argued that invasive species may, not free up defence resources evenly but shift immune defences against well adapted native specialists to defence against more general threats (Joshi and Vrieling, 2005). Another potential adaptation to invasion might be to increase pathogen tolerance. Tolerant individuals alleviated of the fitness consequences of infection could increase pro-invasive behaviours such as dispersal and reproduction. This could have potentially negative impacts on related native fauna through 'pathogen spillback' (Kelly et al., 2009).

All adaptations in immune function made by the invasive host species are constrained by the amount of genetic diversity within the invading population. As invaders often experience population bottlenecks during the introduction process, diversity may be reduced, and vulnerability to infectious disease increased (O'Brien and Evermann, 1988), a concept often associated with agricultural crops (Zhu et al., 2000, Duvick, 1984, Staskawicz et al., 1995). A reduction in haplotype diversity has been observed in D. suzukii, with European populations being the least diverse compared to flies of the native range (Adrion et al., 2014)

2. The virome of Drosophila suzukii, an invasive pest of soft fruit

The work contained in this chapter has been previously published in Virus Evolution, Oxford University Press.

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Simon Fellous and Anne Xuéreb provided samples collected in France 2013.

Fergal Waldron aided with the extraction of RNA from a subset of flies caught in the UK in 2015. He also provided guidance and support for me learning the molecular skills involved in this project.

Madoka Nakai hosted me in Tokyo for the collection of Japanese flies. She was my guide in the field and without her gift of local knowledge, lab space and time Japanese collections would not have been possible.

Jerry Cross was in part responsible, in collaboration with Darren Obbard, for the initial funding proposal for this project. His team of entomologists at East Malling Research, Kent, provided equipment and advice in the collection of UK flies used in this study.

Darren Obbard designed the original bioinformatics pipelines used to identify virus like sequences in metagenomic data and provided feedback on the manuscript as it was prepared for submission and publication.

The published paper is appended to this thesis.

Supplementary data, referred to throughout, is available online as highlighted in the 'Data Availability' section at the end of this chapter.

Introduction

Drosophila suzukii (Matsamura) is an invasive dipteran pest of soft fruit belonging to the subgenus Sophophora. Unusual within the genus, the larvae are well adapted to feeding on ripe fruit still on the plant, adult females possess a heavily sclerotized saw-like ovipositor that allows oviposition under the skin of ripening fruit, and their olfactory system is adapted to respond to fruit rather than microbe volatiles (Karageorgi et al., 2017). These evolutionary innovations may aid the establishment of this species in novel habitats across the globe (Atallah et al., 2014b, Poyet et al., 2015).

First described in Japan in 1916 (Kanzawa, 1935, Matsumura, 1931), *D. suzukii* was reported to be widely distributed in Japan shortly after (Kanzawa, 1939). It was recorded across Asia during the last century (Peng, 1937, Kang and Moon, 1968, Parshad and Duggal, 1965, Okada, 1976, Toda, 1991, Sidorenko, 1992, Amin ud Din et al., 2005), with the first records outside of Asia coming from Hawaii in the 1980's (Kaneshiro, 1983). Since its detection in 2008 in the southern states of the USA (Bolda, 2008) and Spain (Calabria et al., 2012), *D. suzukii* has spread northwards, and was recorded for the first time in the UK in 2012 (Harris and Shaw, 2014). Records now stretch from Sweden (Manduric, 2017) to Argentina (Lue et al., 2017), with secondary invasions thought to be responsible for populations detected in South America and the Indian Ocean Islands (Fraimout et al., 2017).

The damage *D. suzukii* has caused in the fruit growing regions of these countries has driven interest in many aspects of the pest's biology, primarily to improve control methods (Asplen et al., 2015). Conventional chemical control of *D. suzukii* is

challenging because oviposition occurs so close to harvest that management of pesticide residues during crop treatment is of concern (Swoboda-Bhattarai and Burrack, 2015). *D. suzukii* also has a broad host range allowing it to exploit natural refugia, including many wild *Prunus* and *Rubus* spp. (Walsh et al., 2011, Cini et al., 2012, Mitsui et al., 2010, Poyet et al., 2014). An effective biological control agent of *D. suzukii*, compatible with integrated management techniques (Stern et al., 1959), would therefore be highly desirable to horticulturalists worldwide.

Entomopathogenic viruses have the potential for use as environmentally benign, species-specific biological control agents, with certain groups of viruses being used to effectively control insect pests in a range of settings (Hunter-Fujita et al., 1998a). The most successful viral control agents to-date are members of the *Baculoviridae*, with the nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs) finding commercial success against lepidopteran pests in forestry and orchard crops, respectively. These viruses produce polyhedrin occlusion bodies that encase infectious virions during the dispersal stage of the viruses' lifecycle. These protein occlusions protect the virus from environmental degradation, and prolong infectivity in the environment (Bishop et al., 1988, Elgee, 1975). For this reason these viruses have been the focus of viral biopesticide development since their first commercial use in 1975 (Shieh and Bohmfalk, 1980). However, despite the relative success of the *Baculoviridae*, other viral taxa have also been advocated for control purposes. For example: members of the *Nudiviridae,* for use against Rhinoceros beetle (Huger, 2005); a member of the *Reoviridae* for use against Masson pine moth (Peng et al., 2000), and certain viruses of the *Parvoviridae* for use against a range of pests (Bergoin

and Tijssen, 1998). All have shown promise as control agents, despite not achieving commercial success.

As well as identifying some of the natural enemies that could be harnessed to control *D. suzukii* populations, understanding the nature of viral infections in this species may help us better understand the reasons for its geographical spread and establishment (Mitchell and Power, 2003, Torchin et al., 2003, Colautti et al., 2004). In particular, the changing pathogen environment that invasive species encounter in the process of invasion is of interest to the field of ecological immunology, in that relative immune investment is predicted to depend upon the diversity of pathogens experienced its new range (Joshi and Vrieling, 2005, Colautti et al., 2004, Blossey and Notzold, 1995). However, thorough surveys of pathogen diversity in wild invaders remain relatively rare (but see Liu and Stirling, 2006). The genus *Drosophila* is one of the few invertebrate genera in which wild viral pathogen diversity has been explored, with recent virus discovery studies in the genus describing over 50 new viruses (Webster et al., 2016, Webster et al., 2015). Furthermore, a history of intensive investigation of the antiviral immunity of *D. melanogaster* (Xu and Cherry, 2014, Sabin et al., 2010, Huszar and Imler, 2008, Kemp and Imler, 2009, Mussabekova et al., 2017, Bronkhorst and van Rij, 2014, Zambon et al., 2006), means that the viruses of *D. suzukii* may provide a valuable comparative system for the study of immune system evolution.

Here we report the results of a metatransciptomic survey of virus-like sequences associated with *D. suzukii* in both its native (Japanese) and invasive (British and

French) ranges. We describe 18 new RNA viruses, representing 10 different virus families, and confirm their presence in RNA pools using RT-PCR. We place these viruses in the phylogenetic context of recent metatransciptomic studies in the host genus (Webster et al., 2015, Webster et al., 2016) and in invertebrates as a whole (Shi et al., 2016).

Methods

Sample collection

We collected 4450 individual *D. suzukii* across a three-year period between September 2013 and September 2016, including 230 larvae in 2016. We initially focussed on flies in their European invasive range, with sampling subsequently extended to include surveys of flies from native SE Asian range. Flies were collected near Montpellier, France (43.59 N, 3.78 E) in 2013 (collection by AX and SF); in Kent, UK (51.284 N, 0.465 E) during the late summer of 2014, 2015 and 2016 (NCM); and in three locations across Honshu, Japan, during May 2016 (NCM and MN): Tokyo University of Agriculture and Technology, Fuchu (35.683 N, 139.481 E); Naganuma Park, Tokyo (35.637 N, 139.375 E); Shimaminami Shima, Yamagata Prefecture (38.351 N, 140.276 E); Agriculture Total Centre Kaju Research Institute, Fukushima (37.813 N, 140.443 E); and Fuefukigawa Fruit Park, Yamanashi (35.700 N, 138.666 E). We used a combination of commercial bait traps with cotton soaked in a proprietary liquid attractant (DROSO TRAP® and DROS'ATTRACT®, Biobest, Belgium, NV), and a standard sweep net to catch adult flies. Traps, hung at field margin and woodland sites, were collected at intervals of two to three days. All individuals were sorted into vials by trap and species within three hours of collection. We aimed to

morphologically identify all species of *Drosophila* caught (Bächli et al., 2004), however, we also subsequently examined RNA pools for potential contamination due to misidentification. Other species of *Drosophila* were caught in these traps and we collected them together with *D. suzukii*, but they were not analysed further. Wild-collected flies were maintained on solid agar/sugar medium, before being macerated in sterile Ringer's solution (to allow for future experimental virus culture and isolation). In addition to adult fly samples larvae were extracted from infested fruit collected in 2016 from UK and Japan with sterile forceps. Although no *Drosophila* pathogens have previously been reported from the larval stage alone, through their collection we aimed to address the possibility that our sampling method was biased towards mobile adult flies able to respond to attraction based traps.

We pooled trap catches from within a sampling location and immediately extracted RNA from a subsample of the fly (or larva) homogenate using TRIzol® (Invitrogen), before storage at -80°C. We treated pooled RNA samples for possible DNA contamination using DNase (Turbo DNA-free, Ambion) prior to library preparation. To verify RNA quality we tested for contamination using Qubit® and Nanodrop® spectrophotometers. For flies collected in the UK and Japan, library preparation and strand specific sequencing was performed by Edinburgh Genomics (Edinburgh, UK) using Illumina NGS library preparation kits and the Illumina Hi-Seq platform with 120 or 150nt paired end reads. To increase representation of viral and host protein coding RNAs, all libraries underwent depletion of rRNA using Ribo-Zero rRNA Removal Kit (Illumina). Flies collected in France during 2013 were sequenced separately at Beijing Genomics Institute (BGI tech solutions, Hong Kong) using paired-end 90nt reads using

the HiSeq 2000 platform. These libraries underwent Duplex-Specific Thermostable Nuclease (DSN) normalisation and poly-A selection. This process, although enriching for viruses by rRNA depletion, biases virus discovery towards poly-adenylated genomic products only produced by certain viral taxa (e.g. Picornavirales). All raw reads have been submitted to the NCBI sequence read archive under project accession PRJNA402011 (Japan SRR6019484; France SRR6019487; Kent: SRR6019485, SRR6019486, and SRR6019488).

Virus identification and Phylogenetic Analysis

To remove those reads derived from *Drosophila*, we mapped raw reads against the *D. suzukii* genome and transcriptome using Bowtie2 (Langmead and Salzberg, 2012) with the '--very-fast' command-line option. We used Trimmomatic (Bolger et al., 2014) to quality trim and remove adapter sequences from the remaining unmapped raw reads (as pairs) using default parameters, before *de novo* assembly using Trinity version 2.2.0 (Grabherr et al., 2011), retaining a minimum contig length of 500nt. All raw unannotated contigs are provided in supporting file S1 (doi: 10.6084/m9.figshare.5649829). We concatenated all translations of all open reading frames (ORFs) in each resulting contig, and retained only those with an open reading frame of 150 codons or greater. These concatenated protein sequences were used to search against a custom database using Diamond (Buchfink et al., 2015) with a an e-value threshold of 0.01, retaining a single top hit. The target database comprised all of the viral proteins from the Genbank non-redundant protein database ('nr'; Clark et al., 2016), and all of the prokaryote, protist, fungal, nematode, hymenopteran, and dipteran sequences from NCBI refseq protein database. Contigs for which the top hit

was a virus were imported into Geneious®8.0.2 sequence analysis software (Kearse et al., 2012) for manual analysis. We grouped putative virus fragments taxonomically according to their initial best Diamond hit, assembled (Geneious) and manually curated them with reference to closest relatives in Genbank, to give the longest viral sequences consistent with the predicted protein content and structure of that virus taxon.

To infer phylogenetic relationships, we used RNA-dependent RNA polymerase (RdRp) coding sequences unless otherwise stated. The RdRp is generally the most conserved protein across RNA viruses, making it suitable for phylogenetic analysis of this diverse set of virus taxa (Koonin et al., 1993, Shi et al., 2016). RdRp gene sequences were translated and aligned with homologous sequences from their close relatives, as identified by BLAST (Altschul et al., 1990). To align multiple protein sequences we used ClustalW (Thompson et al., 2002) with BLOSSOM cost matrix (Henikoff and Henikoff, 1992). We manually identified regions of poor alignment at the 5' and 3' ends of the alignment and removed them before further analysis. All alignments are provided in supplementary material S2_Data (DOI: 10.6084/m9.figshare.5650117). We then inferred maximum-likelihood phylogenetic trees using PhyML 2.2.3 (Guindon and Gascuel, 2003) with the LG substitution model (Le and Gascuel, 2008). We calculated branch support using the Shimodaira-Hasegawa-like nonparametric version of an approximate likelihood ratio test implemented in PhyML (aLRT; Anisimova et al.,2011). For clarity, the trees presented in figures 2, 4, and 6 are clades from within of larger trees (full trees provided in S3_Data, DOI:

https://doi.org/10.6084/m9.figshare.5650132), realigned and reconstructed using the same methods.

Detection by RT-PCR

To confirm the presence of the newly discovered viruses in original RNA pools we used Reverse Transcription PCR (RT-PCR) to screen for short amplicons of each virus' longest ORF, where possible spanning part of the RdRp gene. We designed primers using the Primer3 (Rozen and Skaletsky, 1999) plugin for Geneious (Kearse et al., 2012), and where necessary manually adjusted oligoes to avoid polymorphic variants identified through pool sequencing, and to avoid synonymous positions at the 3' end. RNA virus sequences identified by metagenomic methods may derive from viral elements endogenised into genomic DNA, if they are expressed (Katzourakis and Gifford, 2010). To test for endogenised viral elements (EVEs) we conducted PCRs (without a reverse transcription step) on nucleic acid samples that contained genomic DNA from the original phenol-chloroform extraction. As these RNA viruses do not produce a DNA intermediate, any viruses detectable by PCR in the DNA fraction are likely to be EVEs.

Virus genome annotation

For viruses with complete, or near complete genomes, we were able to infer genome structure and identify protein functional domains by first identifying ORFs and then comparing these to the Conserved Domain Database with an expected value threshold of 5×10^{-3}, and searching the NCBI 'nr' protein database using BLASTp. Only ORFs of 100 amino acids or longer were annotated, unless notable similarity to closely related viruses was evident. ORFs of less than 200 amino acids that were nested

completely with larger ORFs were disregarded, unless they displayed high similarity to known proteins.

Distribution of RNA sequence reads across samples

To estimate the number of virus reads in each pooled sample, and to detect any cross-species contamination in fly collections, we mapped trimmed forward reads to all new and previously published *Drosophila* virus genomes (including multiple divergent isolates where they were available), a selection of *Drosophila* ribosomal sequences, and a short region of cytochrome oxidase 1 (COI) that has discriminatory power between *Drosophila* species. Sequences were mapped with Bowtie2 (Langmead and Salzberg, 2012) using the '--very-sensitive' option. We report these after normalisation by the number of non-ribosomal reads and the length of each target sequence. We also apply an arbitrary lowest level detection threshold for each putative species of 0.5 total reads per Kb per million non-rRNA reads, to reduce spurious signals caused by low level species contamination, library barcode switching, and cross-mapping to close relatives.

Results

In total, we generated approximately 280 million read pairs, ranging from 33 million pairs (UK - 2016) to 105 million pairs (France - 2013) per library. Our assemblies comprised between 18,431 (Japan - 2016) and 56,384 (UK - 2015) putative transcript contigs. Among these, we identified 18 new RNA viruses associated with *D. suzukii* (Table 2.1.). These viruses represent a variety of RNA virus taxa with positive sense single stranded (+ssRNA), negative sense single stranded (-ssRNA), and double stranded RNA (dsRNA) genomes, and include representatives of the Picornavirales,

Mononegavirales, Bunyavirales, Chuviruses, *Nodaviridae*, *Tombusviridae*, *Reoviridae* and Nidovirales. We did not identify any DNA viruses despite active DNA virus infections being easily detected from RNA sequencing data. We do not report as new any viruses detected in *D. suzukii* that are identical, or near identical (>95% amino acid similarity in the polymerase), to previously published viruses. Those previously described viruses that were detected in *D. suzukii* are detailed in appendices (A.1, also S1_table; DOI: 10.6084/m9.figshare.5650147) and relative read counts in each pool are shown in Figure 2.1.

We have provisionally named these viruses according to the location from which the hosts were sampled. We have chosen not to include taxonomic or host information in the provisional name of the virus, as these are subject to change as phylogenetic relationships

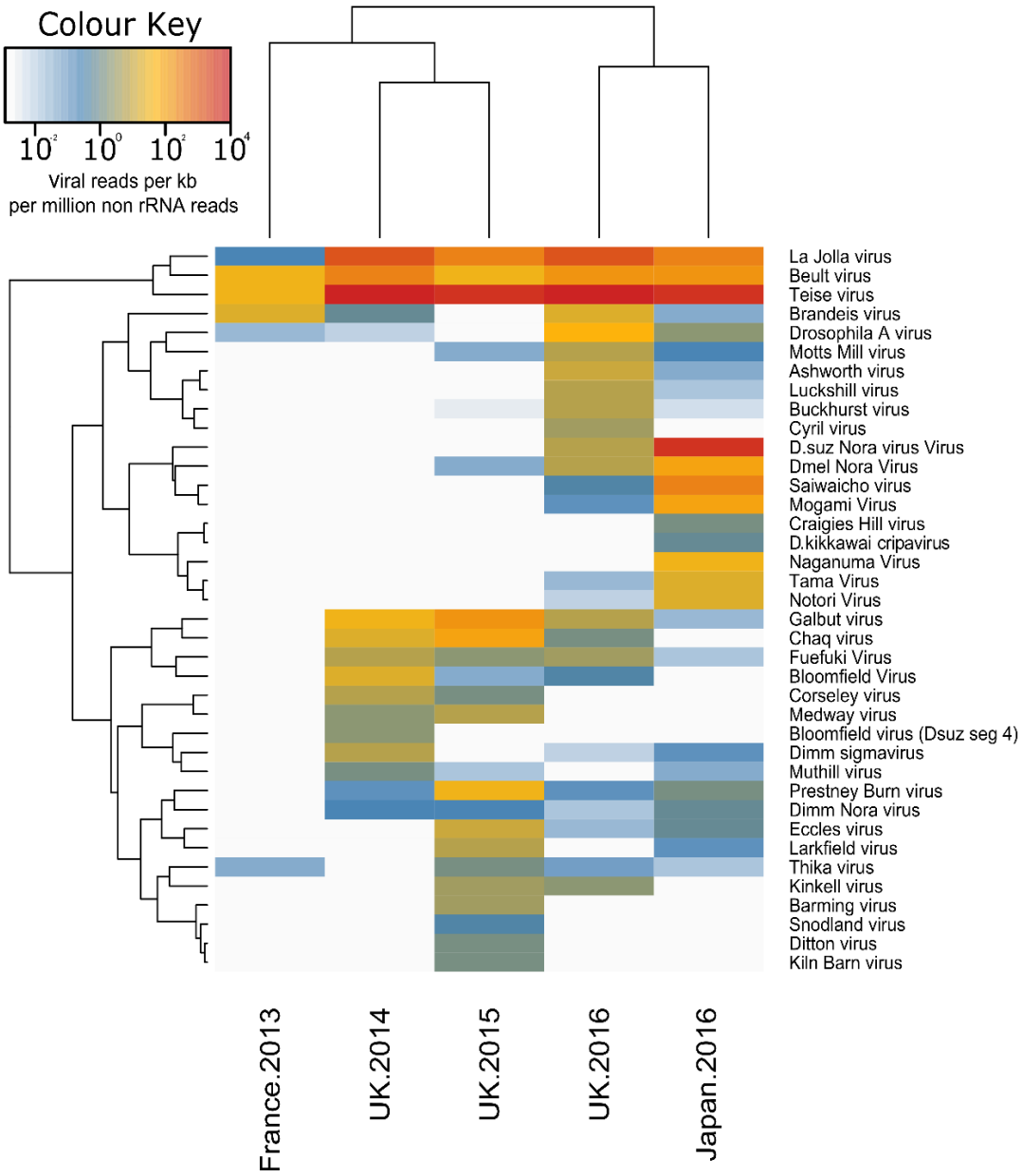

Figure 2.1. The heatmap shows the relative number of reads (log_{10} reads per kb per million non-ribosomal RNA reads) from each library mapping to each of the *Drosophila* viruses. Rows and columns are clustered by their similarity in read frequency on a log_{10} scale. A threshold for detection of 0.5 reads per kb per million non-rRNA reads was applied, however, a small amount of cross mapping is possible between closely related viruses and this may explain the detection of viruses with very low read counts. The low diversity of viruses in the France 2013 sample may be attributable to poly-A selection of RNA libraries. Created using the 'heatmap2' function of the gplots package (Warnes et al., 2016) in R (R Core Team, 2017).

are revised and alternative or additional hosts discovered. The one exception to this rule is *D. suzukii* Nora Virus. This virus is sufficiently closely related to the *D. melanogaster* Nora virus and *D. immigrans* Nora virus that a name outside of this local scheme may cause confusion for future studies. During Phylogenetic analysis, a number of virus-like sequences were identified by BLAST in the public Transcriptome Shotgun Assembly Database (TSA). These have been included in analyses to help improve accuracy of phylogenetic inference, but are not further discussed.

Table 2.1. Novel viruses detected in *D. suzukii*. [a]PCR reactions performed on cDNA. [b]PCR reactions performed on extractions containing nuclear DNA.

Provisional Name	Accession	Host	Taxon	Genome	Longest contig (kb)	Sample(s)	Detected by RT-PCR[a]	Detected by PCR[b]
Beult virus	MF893261, MF893262	Dsuz	Hepe-virga clade	+ssRNA	12	France2013, UK2014, UK2015, UK2016, Japan	+	-
Saiwaicho virus	MF893256	Dsuz	Hepe-virga clade	+ssRNA	10	Japan2016	+	-
Luckshill virus	MF893250	Dsuz	Hepe-virga clade	+ssRNA	3.5	UK2016	+	-
Teise virus	MF893259	Dsuz	*Luteoviridae*	+ssRNA	3.0	France2013, UK2014, UK2015, UK2016, Japan2016	+	-
Tama virus	MF893258	Dsuz	Sobemovirus	+ssRNA	3.5	Japan2016	+	-
Medway virus	MF893251	Dsuz	Sobemovirus	+ssRNA	2.7	UK2014	+	-
Dsuz Nora virus	MF893254	Dsuz	*Picornaviridae*	+ssRNA	12	Japan2016	+	-
Naganuma virus	MF893253	Dsuz	*Nodaviridae*	+ssRNA	1.6	Japan2016	+	-
Fuefuki virus	MF893247	Dsuz	*Nidoviridae*	+ssRNA	16	Japan2016	+	-
Cyril virus	MF893263	Dsuz	Hepe-virga clade	+ssRNA	3.2	UK2016	+	-
Eccles Virus	MF893265-MF893270	Dsuz	*Reoviridae*	dsRNA	4.2	UK2014	+	-
Larkfield virus	MF893249	Dsuz	*Totiviridae*	dsRNA	6	UK2015	+	-
Snodland virus	MF893257	Dsuz	*Totiviridae*	dsRNA	1.6	UK2015	+	-
Mogami virus	MF893252	Dsuz	Chuvirus	-ssRNA	10.5	Japan2016	+	-
Ditton virus	MF893264	Dsuz	*Phasmaviridae*	-ssRNA	7.3	UK2015	+	-
Barming virus	MF893260	Dsuz	*Phleboviridae*	-ssRNA	6.5	UK2016	+	-
Notori virus	MF893255	Dsuz	*Phasmaviridae*	-ssRNA	7	Japan2016	+	-
Kiln Barn virus	MF893248	Dsuz	Chuvirus	-ssRNA	3.7	UK2016	+	-

Viruses with single-stranded positive sense RNA genomes.

Ten of the viruses described here are expected to encode their genomes in +ssRNA. Of these, Teise virus was found at the highest levels across samples. Teise virus is a sobemo-like virus closely related to Prestney Burn virus of *D. subobscura* (Webster et al., 2016) and Motts Mill virus of *D. melanogaster* (Webster et al., 2015), with 90.9% and 88.6% RdRp amino acid similarity respectively (Fig. 2.2, A). The single-stranded positive sense genome of these viruses comprises two unjoined fragments, which may represent subgenomic products (Webster et al., 2015, Shi et al., 2016, Tokarz et al., 2014) (Fig. 2.3), a structure consistent with its close relatives (Shi et al., 2016, Webster et al., 2015). Teise virus is the most geographically widespread virus of *D. suzukii*, with reads appearing in high numbers in both native and naturalised ranges (Fig. 2.1).

Medway virus (Fig. 2.2, B) shares close relationship to Braid Burn virus, previously described from *Drosophila subsilvestris* in the UK (Webster et al., 2016). These viruses belong to a clade of insect viruses distantly related to the Sobemo and Poleroviruses of plants (Shi et al., 2016) . Medway virus appears at low copy-number in our samples with a small number of reads being detected in UK samples from 2014 and 2015. As for other viruses in this section of the Luteo-Sobemo group, the Medway virus genome probably consists of two genomic RNA segments. However, we were unable to detect the second RNA segment and we describe the

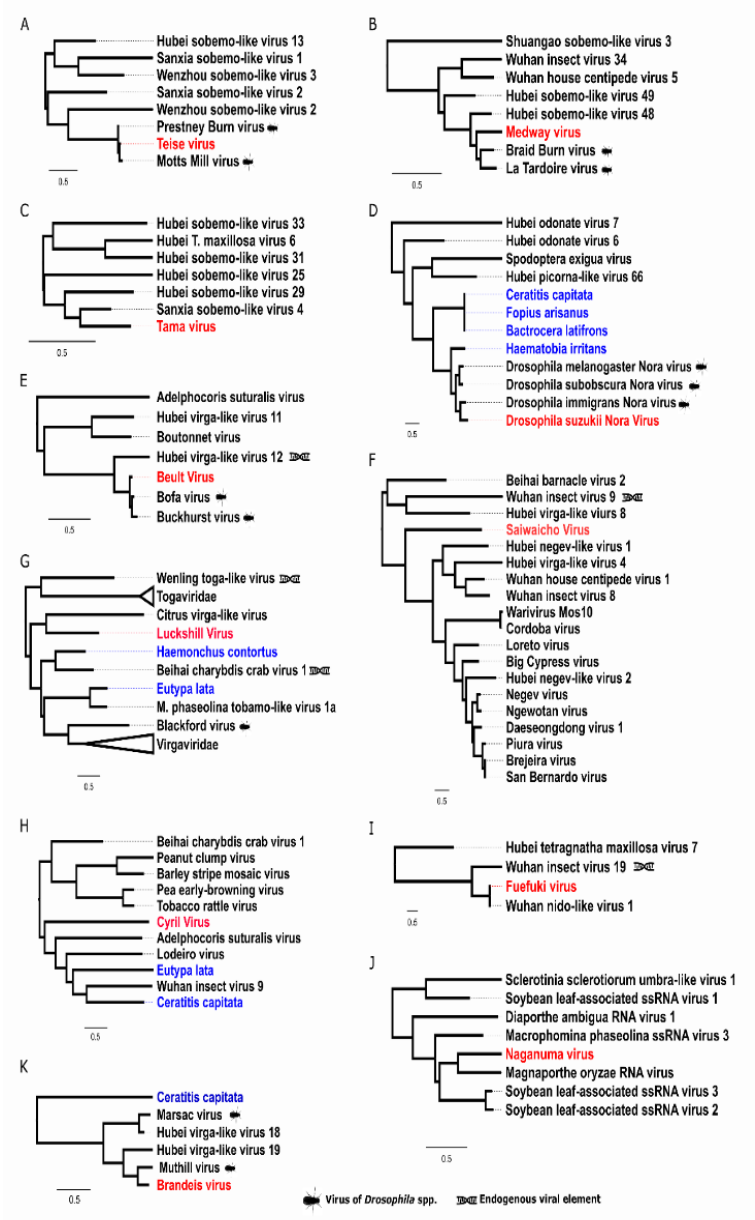

Figure 2.2. Positive sense single stranded RNA viruses. Midpoint-rooted, maximum-likelihood trees were inferred from viral polymerase or viral transferase (H only) sequences. Scale bar represents 0.5 substitutions per site. Putative viruses newly described in association with *D. suzukii* (red) are highlighted alongside virus-like sequences identified in public transcriptome datasets (blue). Viruses previously described as endogenous viral elements are also marked. Tree **A,B,C:** Sobemo-like viruses belonging to clusters within the Luteo-Sobemo clade; **D:** Noraviruses and related cluster of the Picora-Calici clade; **E,F:** Virga-like virus clusters nearby Cilevirus and Negeviruses in the Hepe-Virga clade; **G:** A small cluster of toga-like viruses neighbouring the Alphaviruses, *Togaviridae*; Hepe-virga clade; **H:** A cluster of Virga-like viruses constructed from transferase sequence; **I:** a cluster in the Nidoviruses close to the *Coronaviridae*;. **J:** Cluster of Nodaviruses within the Tombus-Noda clade; **K:** A cluster containing three *Drosophila* viruses within the Hepe-virga clade and distantly related to the *Virgaviridae* and *Bromoviridae*. Complete trees are provided in supporting file S3_data.

virus only from an RNA fragment that contains two ORFs, including the RdRp (Fig. 2.3). Tama virus, a third virus in the Luteo-Sobemo clade (Fig.2.2, C), was only detectable by PCR in Japanese samples.

In our *D. suzukii* collections we detected reads from three separate Nora viruses, *D. melanogaster* Nora Virus (Habayeb et al., 2006), *D. immigrans* Nora Virus (van Mierlo et al., 2014) and the new Nora virus, most closely related to that of *D. immigrans*, but sufficiently divergent from both (37.1% and 30.4% amino-acid divergence at the RdRp locus, respectively) to merit description (Fig. 2.2, D). This clade of viruses also evidently infects other families of 'fruit fly', as they are detectable in the transcriptomes of two species of tephritids (*Bactrocera latifrons* and *Ceratitis capitata*), and can also be found in the transcriptomes of their parasitoid, *Fopius arisanus* (Fig. 2.2, D).

Beult virus was the most geographically widespread virus we identified: we detected Beult virus across sampling locations and years, with reads being especially abundant in samples from the UK in 2014 and Japan in 2016. Belonging to a clade of Virga-like viruses (Fig. 2.2, E), it is very closely related to Bofa virus and Buckhurst virus of *D. melanogaster* and *D. obscura*, respectively (Webster et al., 2016). We identified two different haplotypes of this virus, which share a 98.9% nucleotide similarity: one from the UK, and a second divergent lineage from Japan. Saiwaicho virus (Fig. 2.2, F), closely related to a group of viruses described as Negeviruses by Vasilakis *et al*. (2013), and Luckshill virus (Fig. 2.2, G) belonging to a cluster of viruses with close relationship to the Togaviridae, both also fall within the Hepe-Virga clade of +ssRNA viruses. For

this clade viruses we were able to identify domains for transferases, helicases, and polymerases (Fig. 2.3), with the exception of Cyril virus, which was detected from a fragment of the first large virgavirus ORF, encompassing only transferase and helicase domains. Phylogenetic analysis for this virus was therefore performed using the transferase coding sequence (Fig. 2, H).

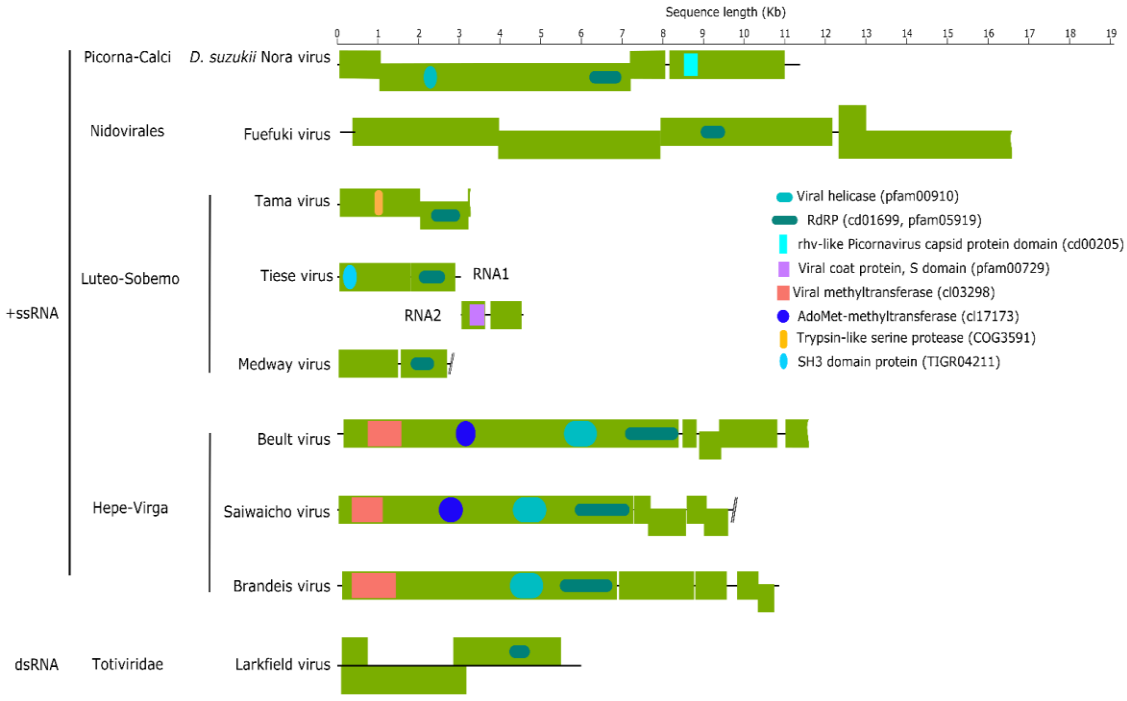

Figure 2.3. The structure of selected dsRNA and +ssRNA virus genomes for which we recover complete or near complete genome sequences. Outer (green) boxes represent boundaries of ORFs and inner boxes represent the relative position of conserved domains identified with reference to the NCBI Conserved Domain Database. Waved outer boxes represent incomplete ORFs and lines ending in slashes represent areas where genome is expected to contain further ORF not recovered from this analysis.

We detected a single Nido-like virus in our samples from the UK and Japan. We have provisionally named this Fuefuki virus, and it has the longest contig recovered for any

of our putative viruses, at over 16.5 kb. Within this near-complete genome we identify five ORFs but only one conserved domain: the RdRp (Fig. 2.3). Fuefuki virus is very closely related to Wuhan nido-like virus 1 (Shi et al., 2016) at 94.8% amino acid similarity in the polymerase. Along with Hubei *Tetragnatha maxillosa* virus 7 and Wuhan insect virus 19 (Shi et al., 2016) these four viruses form a distinct cluster near to the *Coronaviridae*, a family containing some notable vertebrate pathogens, including the SARS virus (Fig. 2.2, I).

Viruses with single-stranded negative sense RNA genomes

Five of the viruses we detected are expected to have -ssRNA genomes. Three of these belong to the Bunya-Arena clade of viruses: Notori virus, Ditton virus, and Barming virus (Fig. 2.4, A-C). Notori and Ditton viruses can be further classified as Phasmaviruses. These were detected in our samples as contigs of around 7kb in length that represent complete, or near-complete L- segments (Bishop and Shope, 1979) (Fig. 2.5). Barming virus, the third putative Bunya-Arena clade virus we identified, belongs to the Phlebo-like cluster of the clade. It too is known from a contig of just over 6kb, also representing the L-segment of the Bunyavirus genome, consisting of one ORF containing the viral RdRp (Fig. 2.5). The closest relative of Barming virus was a viral-like sequence identified in the TSA database from *Colletotrichum cereale*, a plant disease that has been found to cause crown rot anthracnose of turf grass (Crouch et al., 2006).

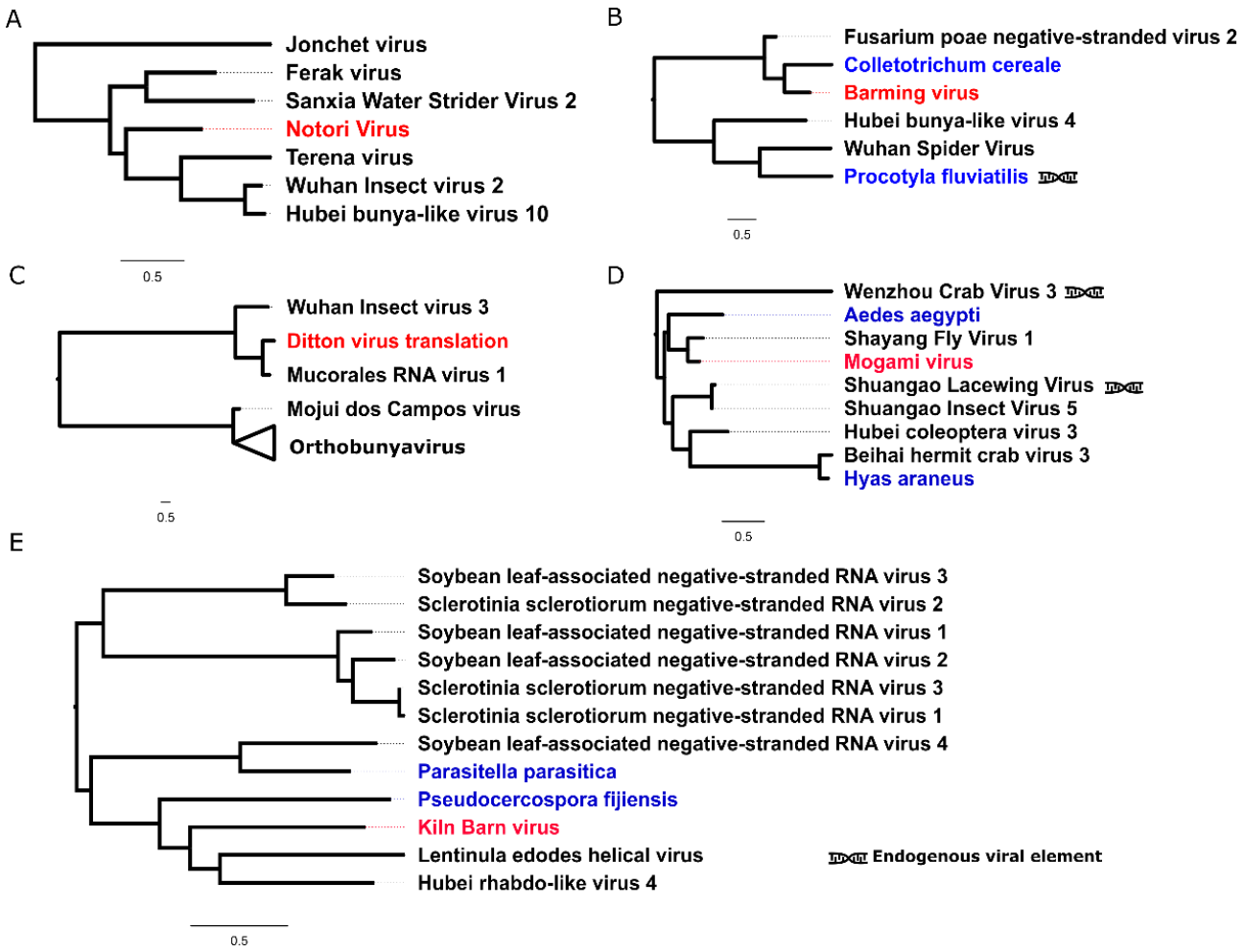

Figure 2.4. Negative sense single-stranded RNA viruses. Midpoint-rooted, maximum-likelihood trees were inferred from viral polymerase sequences. Scale bar represents 0.5 substitutions per site. Viruses newly described in association with *D. suzukii* (red) are highlighted alongside viral-like sequences identified in public transcriptome datasets (blue). Viruses previously described by the original authors as endogenous viral elements are also marked. Tree **A:** Viruses close to Phasmaviruses in the Bunya-Arena group; **B:** Viruses belonging to the Phlebo-like cluster of the Bunya-Arena group; **C:** Orthobunyaviruses (collapsed) and small sister clade consisting of three viruses, including the newly described Ditton virus; **D:** Cluster of the Chuviruses; **E:** Cluster of viruses close to Chuviruses in the Mono-Chu clade. Complete trees are provided in supporting file S3_data.

The remaining -ssRNA viruses we identified belong to the Mono-Chu clade of -ssRNA viruses. From fly samples collected in the UK in 2014 we identified Kiln Barn virus, represented by a 3.7 kb contig containing the RdRp coding domain. The recovery of

this segment allowed phylogenetic analysis and the design of primers for RT-PCR detection, however, the remainder of this virus' genome could not be accurately reassembled and is therefore not annotated in figure 5. Kiln Barn virus clusters phylogenetically with a group of viruses close to the Chuviruses *sensu stricto,* and we find its closest relatives to be Hubei rhabdo-like virus 4 (Shi et al., 2016) and a viral sequence identified in the transcriptome of the Shiitake mushroom fungus *Lentinula edodes* (AGH07920.1). The other virus we identified from this clade, Mogami virus, is closely related to Shayang fly virus 1, a Chuvirus detected in Chinese Diptera (Shi et al., 2016), and was represented by a 10.5kb contig in which from which we are able to identify both glycoprotein and polymerase ORFs.

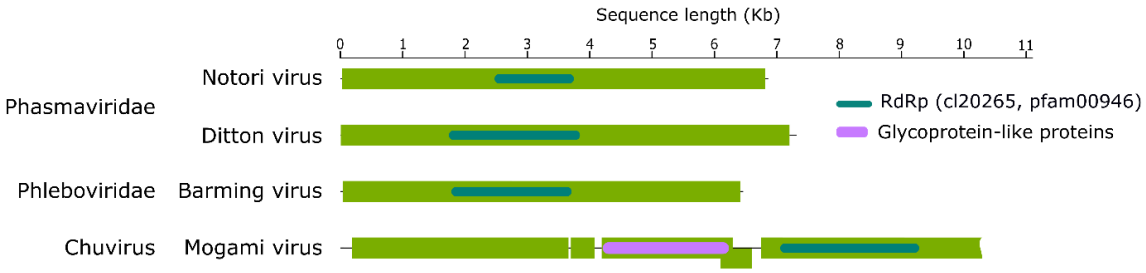

Figure 2.5. The structure of selected -ssRNA virus genomes for which we recover complete or near complete genome sequences. Outer (green) boxes represent boundaries of ORFs and inner boxes represent the relative position of conserved domains identified with reference to NCBI Conserved Domain Database. Waved outer boxes represent incomplete ORFs.

Viruses with double-stranded RNA genomes.

We discovered three viruses predicted to possess double-stranded RNA genomes. These included two Totiviruses, Snodland virus and Larkfield virus, both represented by partial protein coding sequences. Both have closest relatives discovered in insect

pool sequencing by Shi et al. (2016). Larkfield shares a cluster within the Totiviruses which includes a number of ant viruses: two discovered by Koyama et al. (2015) and Koyama et al. (2016) in genus *Camponotus*, and one found here as a virus-like sequence in a published transcriptome of the black garden ant: *Lasius niger* (Fig. 2.6). Its closest relative, Hubei toti-like virus 14, is described as an endogenous viral element (Shi et al., 2016). Snodland virus clusters with a small group of other insect viruses, neighbouring a cluster of mycoviruses associated primarily with powdery mildews (Fig. 2.6).

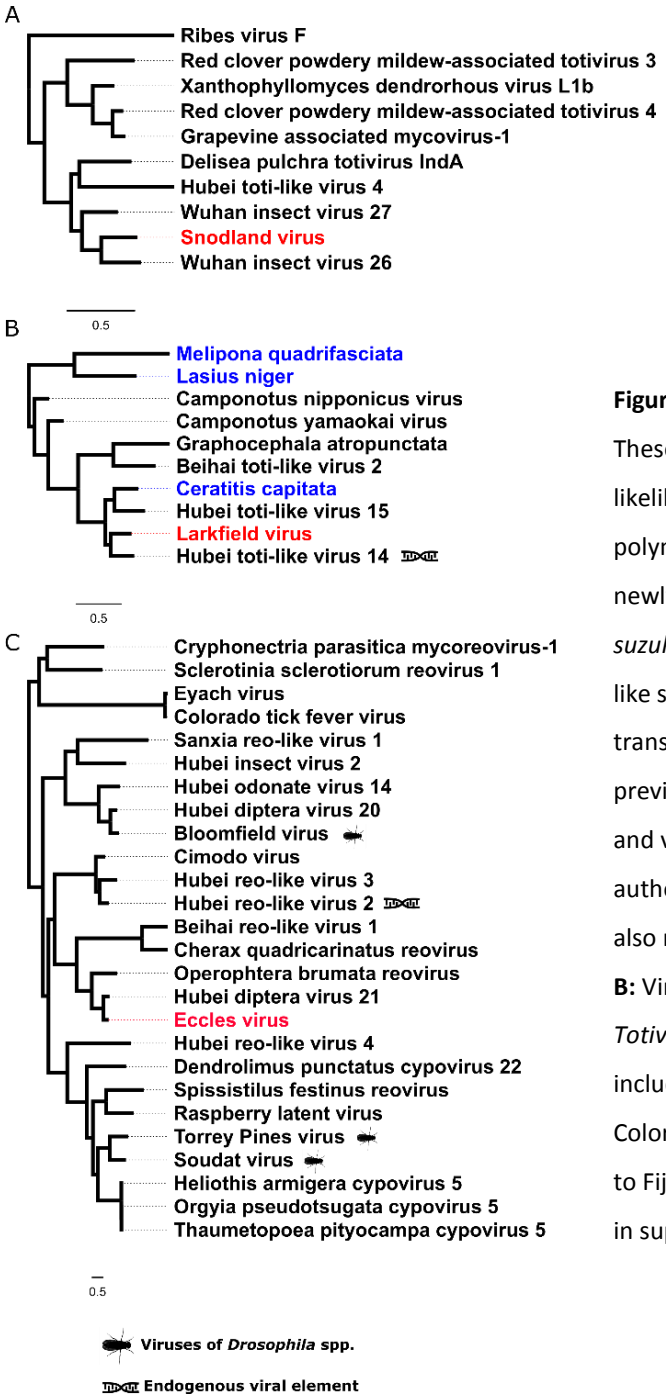

Figure 2.6. Double stranded RNA viruses. These midpoint-rooted, maximum-likelihood trees were inferred from viral polymerase sequences. Putative viruses newly described in association with *D. suzukii* (red) are highlighted alongside viral-like sequences identified in public transcriptome database (blue). Viruses previously described from a *Drosophila* spp. and viruses described by the original authors as endogenous viral elements are also marked. Tree **A:** Totiviruses, *Totiviridae*; **B:** Viruses belonging to a clade of the *Totiviridae*, Toti-Chryso clade; **C:** Reoviruses, including Coltiviruses (Eyach virus and Colorado tick fever virus) and viruses close to Fijiviruses. Complete trees are provided in supporting file S3_data.

The final dsRNA virus identified, Eccles virus, is our only representative of a virus family that has been previously advocated for the biological control of insect pests (Peng et al., 2000): the *Reoviridae*. Eccles virus is most closely related to Hubei Diptera virus 21 (Shi et al., 2016) and a reovirus of the geometrid, *Operophtera*

brumata (Graham et al., 2006). Homology predicts this virus has a multipartite genome consisting of 11 segments, although we were only able to assemble 6 of those segments from our samples.

Known Drosophila viruses

We also detected 18 further viruses previously described from other species of *Drosophila*. Three known viruses were detected at very high levels (below), and are therefore highly likely to represent infections of *D. suzukii.* The first of these is Brandeis virus (MF953177), the genome of which is reported here for the first time (Fig. 2.2). Although originally detected by Webster et al. (2015) in public *D. melanogaster* transcriptome datasets (PRJNA159179; Rodriguez et. al., 2012) and provisionally named, it has not previously been detected in wild flies. It is detected here at high levels (26.8% of all remapped virus reads) in *D. suzukii* samples from France in 2013. Brandeis virus belongs to the Hepe-Virga clade of +ssRNA viruses and is closely related to Muthill virus, a virus previously detected in associated with *D. immigrans* (Webster et al., 2016). We were able to assemble a contig of 10.7 kb, which given homology to closely related virga-like viruses is likely to represent a near-complete genome (Fig. 2.3). The other previously reported *Drosophila* viruses that we reidentified with confidence here are the iflaviruses Kinkell virus and La Jolla virus. Kinkell virus, first described by Webster et al. (2016) was detectable in *D. suzukii* from the UK in 2016, and La Jolla in all samples from all locations. La Jolla virus reads were detected at high abundance in all our samples, comprising up to 30.7% of viral reads in British flies from 2014, and on average 15.0% of virus reads across all samples.

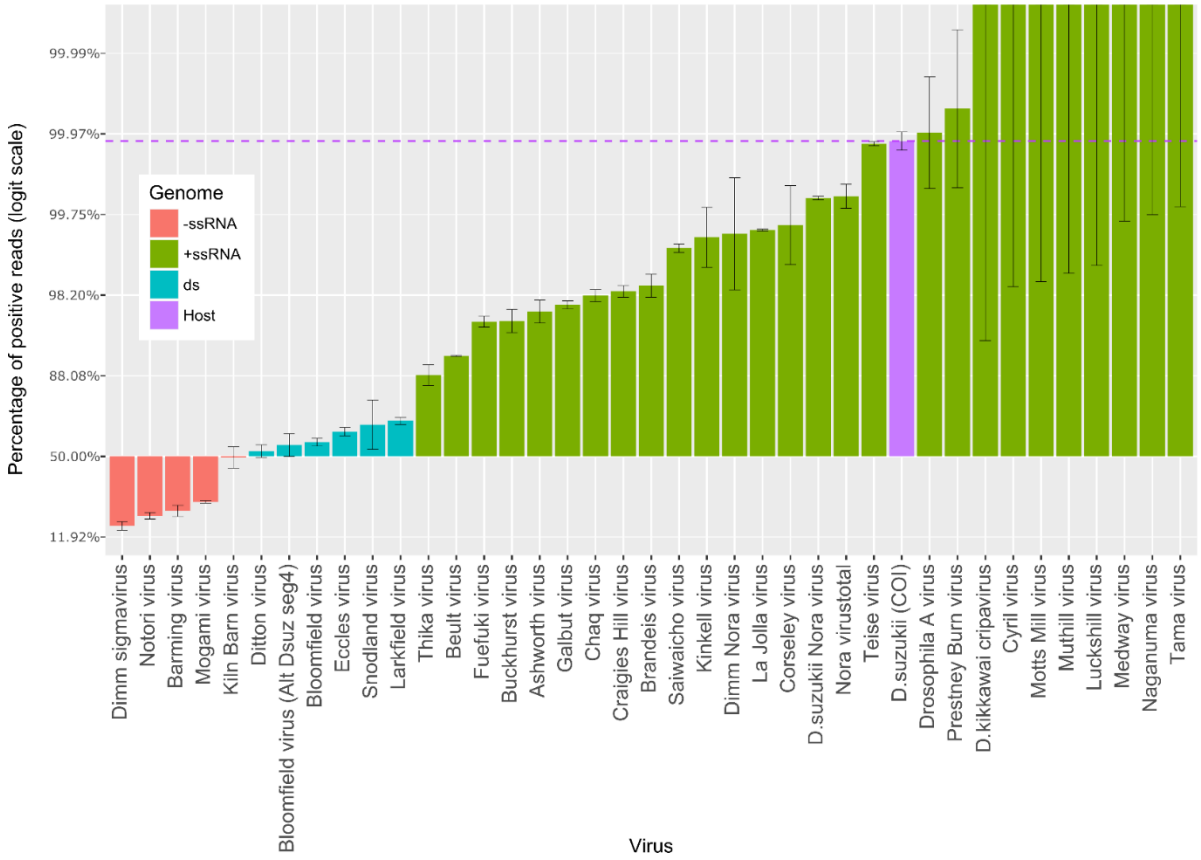

Figure 2.7. Percentage of reads positive sense remapping to virus or host genomes. Percentages presented on logit scale. Bars represent binomial 95% confidence intervals calculated with logit parameterization. Dashed line represents the percentage of positive reads mapping to *D. suzukii* (Host) COI gene.

Four viruses of other *Drosophila* species also appear to be present in *D. suzukii* populations. For example, Corseley virus, a virus most associated with *D. subobscura* (Webster et al., 2016), which was detected at fairly high levels in British caught *D. suzukii* from 2016. It is uncommon in other *Drosophila* species (Webster et al., 2016) and is sufficiently divergent from any newly described *D. suzukii* viruses to minimise cross-mapping of reads. Galbut and Chaq viruses are both known infectious agents of *D. melanogaster*, but appear to be at high levels in 2015 *D. suzukii*. Cross-mapping

to these viruses is unlikely due to their divergence from other *Drosophila* viruses, and host species contamination is unlikely to explain the high numbers of re-mapped reads observed. An unnamed cripavirus of *D. kikkawai* virus reported by Webster et al. (2015) may represent true association for the same reasons. It was detected at low levels in Japanese flies only. Bloomfield virus, a reovirus of *D. melanogaster*, also likely represents true association with *D. suzukii* as we identified a divergent haplotype of one of the 10 genomic segments in *D. suzukii* that has not previously been seen in *D. melanogaster.* It is tempting to speculate that this reflects a history of host shifting and segment reassortment in this virus.

The remaining previously published viruses, were detected at much lower levels (A. 2, also S3_table). Some of these may represent a low level of cross mapping from newly described but closely related viruses. To test this possibility, we remapped short reads identified as mapping to known *Drosophila* viruses back to their close relatives in *D. suzukii*. This identified two instances where notable cross-mapping between known viruses was possible. The few reads mapping to Prestney Burn virus (Webster et al., 2016) are possibly mismapped Teise virus reads, as 1,189 of the 6,400 reads mapping to Prestney Burns virus also align preferentially to two specific regions of the Teise RNA 1 fragment. Similarly, 27,650 of 90,928 reads mapping to *D. melanogaster* Nora virus also align to the *D. suzukii* Nora virus. In addition, a number of reads may result from sample contamination by misidentified flies and/or library cross-contamination (such as barcode-switching, see: Sinha et al., 2017; Kircher et al., 2011; and Ballenghein et al., 2017). This includes viruses with no close relative associated with *D. suzukii*, such as Thika virus, Craigies Hill virus and Ashworth virus

(*unpublished*), or viruses with biologically constrained host ranges, such as the Sigma viruses, along with *Drosophila* A virus (DAV), Drosophila C virus (DCV), and *D. melanogaster* Nora virus that were known to be present in *D. melanogaster* samples run alongside the 2016 *D. suzukii* samples.

Virus abundance and composition varies among samples

To estimate the amount of virus in each of our samples we mapped all raw reads back to new and previously published putative *Drosophila* virus genomes (Fig. 2.1). The percentage of non-rRNA reads that mapped to any *Drosophila* virus varied from 0.09% in the poly-A selected French sample up to 5.14% in UK sample from 2016, with an average of 4.27% of reads being viral in Japanese and British pools. Remapping of reads generated by strand specific sequencing (British and Japanese samples), showed that all viruses with negative sense genomes were represented by between 15% and 49% positive sense reads; viruses with double stranded RNA genomes by 53.3% to 70.8% positive reads; and positive sense viruses 88.3% to 100% positive reads (Fig. 2.7). The only positive sense ssRNA viruses that lacked negative sense reads were represented by less than 2000 reads in total, although in some cases the proportion of negative sense reads was very low. These included Teise virus and La Jolla virus, which displayed extremely large numbers of reads (3,974,042 and 1,326,799 respectively) and the latter of which is a confirmed infectious agent of Drosophila (Webster et al., 2015).

The virus composition varied markedly among samples from different times and locations (Fig. 2.1). Six of the newly described viruses were probably only present in Japanese samples: Mogami virus, Notori virus, Naganuma virus, Saiwaicho virus,

Tama virus, and *D. suzukii* Nora virus, whereas many of the new and previously described viruses are found only in the fly's invasive range and are absent, or at negligible levels, in native, Japanese samples. Despite applying a detection threshold for very low viral read numbers, there are several sources of error when attempting to analyse patterns of virus sharing among years or sampling locations. For example, barcode switching (Sinha et al., 2017, Kircher et al., 2011, Ballenghien et al., 2017) and other sources of cross-contamination between libraries sequenced together on the Illumina platform may allow miss-assignment of reads between the Japanese and British samples from 2016, and also from other drosophilid libraries analysed at the same time. Furthermore, cytochrome oxidase read mapping suggests a small proportion of contaminating reads deriving from *D. melanogaster* and *D. immigrans* were present in some of our datasets. For example, in the Japanese sample of 2016 1.3% of COI reads mapped to *D. immigrans* (potentially misidentified larvae) and in the UK sample of 2015 0.74% of reads mapped to *D. melanogaster*. The *D. melanogaster* reads may represent misidentification or cross-mapping, as the species are quite closely related, but it is more likely that they are the result of contamination across libraries through barcode switching as *D. suzukii* samples were sequenced in parallel with unrelated drosophilid libraries.

Discussion

Here we make a first survey of the viruses associated with the invasive *Drosophila* pest *D. suzukii* in its native and invasive ranges. Alongside 18 new viruses, not previously described from any organism, we confidently identified a further seven viruses associated with this novel invasive host that had previously been described

from other *Drosophila* species. Some novel viruses were detected solely from the native range of *D. suzukii* and others from the invasive range, but rarely from both habitats.

These viruses were identified from metagenomic sequencing of samples of wild *D. suzukii*. Although their presence as RNA but not DNA implies that they are not expressed endogenised viral elements (i.e. EVEs), it remains possible that some are not truly infections in this fly, but may be contaminants of the surface of the fly or infect a commensal, pathogen, or food organism within the fly's gut lumen. However, we believe that this is unlikely to be the case for most sequences, as previous studies that additionally used the presence of virus-derived 21nt short interfering RNAs to demonstrate active replication (Webster et al., 2015) found that the majority of viruses identified in similar metatransciptomic sequencing of *D. melanogaster* constituted active infections. For most of these viruses active replication is further supported by the relative proportions of positive and negative sense reads mapping to each virus. Although the exact ratio of positive to negative strand RNA is known to fluctuate through the course of infection (Martínez et al., 2011; Thébaud et al., 2009), all viral read counts deviated from the ratio expected if no replication was occurring (Fig 2.7). This was unambiguous for all of the -ssRNA viruses and dsRNA viruses, which showed substantial numbers of the positive sense sequences required for protein synthesis and replication, and strongly supportive for most +ssRNA viruses, almost all of which displayed some of the negative sense reads expected from replication intermediates. There is also a possibility that cross-species contamination or

barcode-switching could result in spurious host allocation, but this is not compatible with the read number or distribution of reads for the majority of viruses (above).

In addition, recent large-scale invertebrate virus discovery projects (Shi et al., 2016) give us a greatly increased confidence in the phylogenetic relationships of newly identified virus sequences. In particular, although some virus taxa have a diverse host range, it seems reasonable to infer that *D. suzukii* is the true host for viruses with very close relatives confirmed to infect another insect. For example, Mogami virus (Chuvirus) is distantly related to any known *Drosophila* virus, but is closely related to Shayang Fly virus 1 (Shi et al., 2016) and clusters within a group of viruses that are only described from insect samples (see Fig. 2.4, D). Nevertheless, this pattern is not true for all viruses described here. Specifically, two of the 18 novel viruses in this study (Ditton virus and Barming virus), are more closely related to Mycoviruses than they are to any entomopathogenic viruses and one (Luckshill virus) is most closely related to a sequence found in a parasitic nematode of ruminants. And, while this pattern does not exclude the possibility of these being true viruses of *D. suzukii*—as many viral families contain a broad range of hosts including those of different phyla and patterns of host switching are still little understood—these are among the best candidates to be infections of *Drosophila* parasites or gut fauna, rather than *D. suzukii* itself.

The potential for these viruses to be used as biological control agents is currently unclear. Commercially successful viral biocontrol agents have in the past only come from the dsDNA virus family *Baculoviridae*, which was not represented in our

collections, and most lineages represented here have not been investigated for their ability to be cultured and applied as control agents. Indeed, few viruses in the identified families have been successfully isolated for experimentation, and many are known only from metagenomic sequencing. The only virus family we found in associated with *D. suzukii* that has any history as a control agent (Zeddam et al., 2003b, Peng et al., 1998, Peng et al., 2000) is the reovirus 'Eccles virus'. Eccles virus was relatively rare in our samples, but this may speak to the potential pathogenicity of the virus, as flies harbouring a particularly pathogenic virus, especially one that has a short latency period, may be less likely to visit baited traps (Gupta et al., 2017). Further investigation of this virus, including isolation and pathogenicity assays, are needed before any further conclusions can be drawn about its utility as a control agent. Viruses potentially lethal to *D. suzukii* may also await discovery in other species of *Drosophila.* Indeed, pathogens have the potential to display increased virulence following a host shift event (Longdon et al., 2015) and the susceptibility of *D. suzukii* to viruses of *D. melanogaster* has been shown experimentally (Cattel et al., 2016, Lee and Vilcinskas, 2017). Here we show the potential association of viruses from *D. melanogaster*, *D. immigrans* and *D. subobscura* with *D. suzukii* in the wild. Further investigation of the viral community experienced by many different *Drosophila* in nature may, therefore, be of both academic and applied interest.

Given our focus on an invasive species, the potential for a shift in the virological environment associated with invasion is of particular interest. Theory predicts that organisms may experience a 'release' from natural enemies, including pathogens, in their invasive range due to low host densities and founder effects at the invasive edge

(Keane and Crawley, 2002): However, this idea remains contentious, as supporting evidence is limited (Colautti et al., 2004). It has also been hypothesised that invasives, rather than experience a drop in overall number of enemies, undergo a shift in the type of enemy encountered, from co-evolved specialists in the native range to more generalist enemies, quickly able to adapt to a new host, in the naturalized range (Joshi and Vrieling, 2005). In this study, we do detect an apparently marked difference in the virus communities of flies from different areas within its expanding geographical range. Although a low level of species contamination in certain pools means that these findings should be treated with some caution, five of the new viruses described (Saiwaicho virus, Tama virus, Mogami virus, Naganuma virus and Notori virus) were only detected at high levels in Japanese (native) flies. These five viruses are not particularly closely related to any previously described *Drosophila* viruses (Fig. 2.2 and Fig. 2.4) and may represent a more specialized relationship with *D. suzukii*. In contrast, the three most ubiquitous viruses across all samples, La Jolla virus, Teise virus and Beult virus are either a known generalist (La Jolla) or very closely related to a virus in another related hosts (Fig.2.2, A and E). If confirmed, this pattern could reflect a shift in natural enemy type from native to invasive range of *D. suzukii*.

Data availability

All raw unannotated contigs are provided in supporting file S1_data (DOI: 10.6084/m9.figshare.5649829); all alignments are provided in supplementary material S2_data (DOI: 10.6084/m9.figshare.5650117); all large phylogenetic trees from which figures are taken are available in S3_data (DOI: 10.6084/m9.figshare.5650132); a table of known *Drosophila* viruses detected in *D.*

suzukii is available in S1_table (DOI: 10.6084/m9.figshare.5650147); a table of PCR primers used for virus detection is in S2_table (DOI: 10.6084/m9.figshare.5650156); Number of reads mapping to all known drosophila viruses and to novel D. suzukii viruses is in S3_table (DOI: 10.6084/m9.figshare.5830644). Coverage depth graphs for novel virus genomes are shown in S1_figure (DOI: 10.6084/m9.figshare.5893324).

All raw reads have been submitted to the NCBI sequence read archive under project accession PRJNA402011 (Japan SRR6019484; France SRR6019487; Kent: SRR6019485, SRR6019486, and SRR6019488). All novel virus genomes are submitted separately to the NCBI sequence read archive under the accessions outlined in table 1.

3. The Prevalence and Host Range of Drosophila Viruses

This chapter is all my own work but will be revised for submission as a manuscript with Darren Obbard as a second author.

Darren Obbard has provided feedback on parts of this manuscript and will continue to do so in preparation for submission.

The analysis in this chapter is adapted from that used in Webster et al. (2015) and R scripts from this analysis were provided courtesy of Darren Obbard.

Introduction

Most viruses are capable of infecting more than one host (Pedersen et al., 2005, Taylor et al., 2001, Cleaveland et al., 2001). The number of hosts a virus can exploit and what influences the ability of a virus to infect multiple hosts is of great interest, not least because some of the most deadly human pathogens are recent shifts from other animal hosts: zoonosis (Morens et al., 2004, Woolhouse et al., 2005). Understanding the patterns of virus host specificity in wild animal populations may lead to greater ability to predict and control such zoonotic outbreaks in human and livestock populations.

Of the viruses capable of infecting multiple hosts some are able not only to infect, but also to transmit between host species, constituting true multi-host pathogens as opposed to those constituting pathogen spillover and only capable of dead-end infection (Woolhouse et al., 2005, Cleaveland et al., 2007). Defining what is meant by these terms is essential for further discussion of multi-host systems (Funk et al., 2013). Fenton and Pedersen (2005) present a useful framework for classifying types of multi-host-pathogen interactions. They describe four categories of multi-host pathogen and associated infection outcome delimited by their within and between species transmission rates. The first being 'spillover', where the transmission rate between an endemic host and a second, recipient species, and the transmission rate within that recipient species, are both low. This results in rare and transient infections of the recipient population. For example, West Nile encephalitis (Campbell et al., 2002) which is transmitted in rare cases from birds to humans but is incapable of continued transmission between human hosts. Infections by these relative specialists will only

be detected at low prevalence in hosts other than the natural endemic due to this low transmissibility. Secondly, 'apparently multi-host pathogens' are transmitted at a high rate from their endemic host to a recipient host but low transmission within that recipient species prevents spread and persistent infections. The endemic species acts as a reservoir allowing the repeated reinfection of the susceptible recipient host. Potentially high prevalence of these pathogens in the recipient host can give the appearance of a true multi-host pathogen, however, like cases of spillover these infections are non-persistent in recipient species populations. Rabies is a notable example of such a dynamic: infections in the recipient host, humans, are relatively frequent but a lack of transmission between human hosts prohibits persistent infections in human populations (Nel and Markotter, 2007). Thirdly, 'true multi-host pathogens'. These pathogens have high transmission rates between endemic hosts and recipient hosts but are also able to be transmitted at high rates within recipient host populations. These pathogens, such as brucellosis infection in cattle, bison and elk (Rhyan et al., 2013) are able to persist in either host population independently and represent true generalists. Finally, 'potential emerging infectious disease' are defined as pathogens able to transmit well within recipient host populations but that are rarely transmitted between host species. Recent human outbreaks of Ebola or SARS can be classified in this way.

The barrier to infection in these cases may be ecological or geographical meaning that changes in species range or anthropogenic disturbance, resulting in novel contact between species, could result in the emergence of new infections and disease. Indeed, disease emergence has been associated with changes in host geographical

range in a range of different taxa (Lips et al., 2006, Vasilakis and Weaver, 2008, Stricker et al., 2016, Jones and Coutts, 2015, Karesh et al., 2012, Patz et al., 2004). One increasingly common way in which populations are exposed to novel sympatric taxa is through human mediated biological invasions. These introductions grant certain species access to new habitats inaccessible before the activities of modern man (Chapman et al., 2017). Once introduced, these populations are likely to encounter populations of closely related native species through shared or overlapping resource requirements. At this point there is potential for species-species transmission of disease novel to either the invading or invaded population.

If contact between an infected endemic host and a potential recipient species is established, the likelihood of transmission, replication and onward transmission is dependent on the host immune response and the parasite's ability to overcome it (Parrish et al., 2008). The chances of a parasite circumventing the host immune system are increased if host and parasite share some evolutionary history, i.e. infection success increases as a function of relatedness between host species (Engelstädter and Hurst, 2006, Cooper et al., 2012, De Vienne et al., 2009, Hadfield et al., 2014). This 'phylogenetic distance effect' has been demonstrated in a number of different experimental host-parasite systems (Longdon et al., 2011a, Perlman and Jaenike, 2003) and through surveys of parasite incidence in related wild hosts (Pedersen et al., 2005, Antonovics et al., 2002, Davies and Pedersen, 2008, Ricklefs and Fallon, 2002). Experimental studies benefit from the ability to measure the effect of phylogenetic distance on infection success post-transmission but do not reflect the relative transmission likelihood of pathogens as effected by variation in the ecology

or geographic range. Wild host-parasite surveys on the other hand, encompass these natural causes of transmission but often lack information about infection outcomes in hosts, lowering the ability to distinguish between apparent multi-host pathogens and true multihost pathogens, and between pathogen spillover and emerging pathogens. Model host-parasite systems that can be studied in both of these ways, therefore, provide a powerful synthesis allowing us to understand the relative importance of immunological and ecological factors influencing parasite host range.

A wild model system that potentially allows this combination are fruit flies of the genus *Drosophila* and their natural pathogens. An increasingly clear picture of *Drosophila* parasite diversity, especially that of viruses, is emerging through successive metatransciptomic studies (Webster et al., 2016, Webster et al., 2015, Medd et al., 2018, Palmer et al., 2018b, Shi et al., 2018). Alongside wild observations, *Drosophila melanogaster's* status as established model species in immunology grants unprecedented understanding of the immune function of the genus (Alarco et al., 2004, Hultmark, 1993, Hoffmann and Reichhart, 2002). Host switching specifically, has also received attention in this genus with studies on Drosophila Sigma Virus (Longdon et al., 2011b) and Drosophila Nora Virus (van Mierlo et al., 2014) revealing the mechanics of host specialisation and the molecular barriers to cross-species transmission. Furthermore, a number species of *Drosophila* are human commensals or invasive species with well tracked recent geographical range changes (David and Capy, 1988, Beckenbach and Prevosti, 1986, Fraimout et al., 2017). One such species, *Drosophila suzukii*, is an oriental species currently considered invasive in the Western Palearctic, Nearctic and Neotropical regions. Its relocation, probably aided by human

activity (Fraimout et al., 2017) has brought it into recent contact with a selection of congeners that share the same wild fruit recourses (Rombaut et al., 2017). This *Drosophila* community therefore represents an excellent opportunity to study the incidence of pathogens within a community recently effected by swift range change, a situation hitherto poorly studied.

The shift of native parasites into invasive hosts or vice versa, not only gives an opportunity to study disease emergence dynamics in the wild, its occurrence could also explain the relative success of certain biological invasion events. Small vanguard populations may, through founder effects, experience a form of 'enemy release' (Keane and Crawley, 2002, Mitchell and Power, 2003), allowing them to exploit new habitats with a reduced parasite burden compared to native conspecifics. This easement in enemy pressure could in theory lead to the success of individuals that divestment away from costly immune function and into more 'invasive' traits such as reproduction and dispersal (White and Perkins, 2012, Torchin et al., 2003, Horrocks et al., 2011, Zuk and Stoehr, 2002). However, the evidence for reduced parasite burden in invasive populations is scant (Colautti et al., 2004) and generally constrained to a limited number of parasites or predators (Phillips et al. 2010). This theory further assumes the relative inability of 'enemies' in the invaded range to attack the newly invaded species. However, depending on rates of exposure and phylogenetic distance, it possible that rather than an overall decrease in the number of enemies, an invasive species could experience a shift from a pathome consisting of a mixture of co-evolved specialists and generalists to one overpopulated by generalists able to shift from native hosts (Joshi and Vrieling, 2005). Indeed, quickly

evolving enemies such as RNA viruses in the invaded range my infect the new host quickly upon arrival (Faillace et al., 2017) and cause increased pathogenicity following this host shift event (Longdon et al., 2015).

Here, we study the occurrence of 21 viruses in five co-occurring *Drosophila* species, one of which is the recent invasive, *D. suzukii*. This survey estimates the prevalence of each virus in all hosts in a range of sampling locations across three sampling years in two countries. Viruses first described from each of the five species are included and allow us to assess host range and potential recent host switching events as well as an approximation of pathogen burden in native and invasive populations of *D. suzukii*.

Methods

Sample Collection and Identification

We collected 5826 individual flies of five different *Drosophila* species: 2459 *D. suzukii*, 719 *D. melanogaster*, 1494 *D. immigrans*, 560 *D. subobscura*, and 65 *D. obscura*. We collected British flies in Kent, UK (51.284 N, 0.465 E) during late August and September of 2014, 2015, and 2016, and Japanese flies in three locations across Honshu, Japan, during May 2016: Tokyo University of Agriculture and Technology, Fuchu (35.683 N, 139.481 E); Naganuma Park, Tokyo (35.637 N, 139.375 E); Shimaminami Shima, Yamagata Prefecture (38.351 N, 140.276 E); Agriculture Total Centre Kaju Research Institute, Fukushima (37.813 N, 140.443 E); and Fuefukigawa Fruit Park, Yamanashi (35.700 N, 138.666 E). Samples of *D. suzukii* are the same samples as appear in chapter 2 (Medd et al., 2018) but expanded to include all species caught at these locations using the same methods. We used a combination of

commercial bait traps with cotton soaked in a proprietary liquid attractant (DROSO TRAP® and DROS'ATTRACT®, Biobest, Belgium, NV), and a standard sweep net to catch adult flies. Traps, hung at field margin and woodland sites, were collected at intervals of 2–3 days. All individuals were sorted into small pools by trap and species within 3 h of collection. We morphologically identified all species of *Drosophila* caught (Bächli et al. 2004) checking for species cross contamination in larger pools through NGS see: Medd et al. (2018) . Flies were then kept on hard agar food medium before being macerated in TRIzol® (Invitrogen) and immediately stored at - 80°C. In addition to adult fly samples, larvae were extracted from infested fruit collected in 2016 from UK and Japan with sterile forceps. Although no *Drosophila* pathogens have previously been reported from the larval stage alone, through their collection we aimed to address the possibility that our sampling method was biased towards mobile adult flies able to respond to attraction based traps.

Estimation of Viral Prevalence

We used reverse transcription polymerase chain reaction (RT-PCR) to survey trap pools for the presence of 21 previously published (Medd et al., 2018, Webster et al., 2016, Webster et al., 2015) *Drosophila* viruses (Beult virus, Brandeis virus, Chaq virus, Charvil virus, Cherry Gardens virus, Craigies Hill virus, *Drosophila* A virus, *Drosophila* C virus, Eccles virus, Galbut virus, Kallithea virus, Kinkell virus, La Jolla virus, Larkfield virus, Medway virus, Motts Mill virus, Muthill virus, Presney Burn virus, Snodland virus, Teise virus, Thika virus). We extracted RNA from each pool of fly homogenate using TRIzol® (Invitrogen) and progressed to reverse transcription using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega). cDNA

generated in this step was then stored at -20°C until further use. We used polymerase chain reaction (PCR) to identify pools containing virus cDNA. Oligonucleotide primers and conditions used in each virus assay were as published at the time of their respective discoveries (Webster et al., 2016, Webster et al., 2015, Medd et al., 2018) and are also included in the appendix (B.1). The integrity of cDNA was regularly checked using primers amplifying a region of arthropod 28S ribosomal DNA sequence (Arth_28s_F:TACCGTGAGGGAAAGTTGAAA, Arth_28s_R: AGACTCCTTGGTCCGTGTTT). PCR's were conducted using BIOTAQ™ DNA polymerase (Bioline) and products visualised by electrophoresis run on 1% agarose gels. Samples were scored as positive on the presence of a band appropriate to the expected size of amplicon for that particular assay. Positive controls for viruses consisted of original discovery pools (Medd et al., 2018, Webster et al., 2016, Webster et al., 2015) reliably giving positive results for each assay.

Because samples represented pools of between 1 and 50 flies positive results in pools do not directly reflect the number of flies carrying a particular virus. Therefore, to infer underlying viral prevalence we used a maximum likelihood approach (Webster et al., 2016, Webster et al., 2015) that assumes an underlying prevalence for each species-location combination and that the number of infected flies observed is binomially distributed given this assumed prevalence. Then, given the number of flies included in each small pool, and the number of small pools or single flies that test positive, we searched across the range of possible prevalence values (zero to one) identifying the prevalence which maximised the likelihood of the observed set of positive/negative PCR assays.

To analyse the change in prevalence between samples we summed the likelihood estimates of each virus-host combination separately for each of individual sampling location-year combination, giving the likelihood that each location-year combination exhibits a different ML prevalence. Then taking the sum of the binary presence/absence data for those location-year combinations, we estimated the ML prevalence again, giving the likelihood for those samples having the same prevalence. We then used a likelihood ratio test to ask which model is most likely, given that there are two extra parameters. All statistical analysis and data visualisation was conducted in R v.3.5.1 (R Core Team, 2017) using the 'stats' and 'ggplot2' packages respectively.

Results

Viral Prevalence

Considering all species in all sampling locations and years, a mean of 28.78% of flies tested positive for at least one of the 21 viruses in our study. The most frequently infected species being *D. subobscura*: of which 77% of flies sampled were infected with at least one virus. The species with the lowest virus prevalence was *D. immigrans* with only 1.7% of flies testing positive for at least one virus. *Drosophila suzukii* had an overall infection prevalence of 39.89% higher than that of *D. melanogaster* at 22.52%. The species with the highest recorded diversity of infections was *D. suzukii*, testing positive for 15/21 in total and harbouring an average diversity of 7.25 viruses per sample site, however, if corrected for by the number of individual flies caught *D. obscura* showed the highest diversity, with an across sample site mean of 0.041 viruses per fly compared to *D. suzukii* at 0.013 viruses per fly. The number of different

viruses detected per fly in this study does not necessarily represent the overall diversity of viruses harboured by each fly species as the particular viruses assayed for are an arbitrary subset of the Drosophila virome.

Of these 21 viruses surveyed, three viruses were not detected in any sample pool: DAV, Craigies Hill virus, and Kallithea virus. These viruses, all originally discovered in *D. melanogaster* are known to occur at relatively low prevalence (<7%) in their endemic host. Of the remaining 18 viruses found in at least one pool of flies from any species, no virus was found infecting more than two hosts. In these 14 'multi-host' viruses there was often a substantial difference between the prevalence in the assumed native host, the species from which the virus was first described, and the second, alternative, host (table 3.1). In viruses where prevalence is higher in the assumed native host, 6/14 multi-host viruses, prevalence is between 10.6 fold and 1893.3 fold (mean: 416.1 fold) higher than in the recorded alternative host. In seven of these viruses prevalence was higher in a species other than its assumed native host. Eccles virus, first described from British populations of *D. suzukii* occurs at a substantially greater prevalence in *D. subobscura* than it does in *D. suzukii*: 38.4% and 9.52%, respectively. A similar pattern can be seen in Muthill virus, Larkfield virus, Medway virus, all occurring more frequently in a host other than that from which they were originally described. This is also true of Charvil virus, La Jolla virus, and Brandeis virus, although these were not detected at all in their assumed native host and only at very low levels (<0.25%) in their alternative host (table 3.1).

Table 3.1. The mean global prevalence (bold) of viruses detected at any prevalence in assumed endemic and alternative hosts. Values in parenthesis are 2-log likelihood intervals for prevalence estimates.

	Classification	Assumed endemic host	Mean prevalence in assumed endemic host (%)	Assumed alternative Host	Mean prevalence in assumed alternative host (%)
Muthill virus	Hepe-Virga	*D. immigrans*	**1.45**(0.39-3.87)	*D. suzukii*	**7.78**(5.63-10.47)
Charvil virus	Flavi	*D. melanogaster*	**0**	*D. immigrans*	**0.03**(<0.01-1.16)
Brandeis virus	Hepe-Virga	*D. melanogaster*	**0**	*D. suzukii*	**0.06**(<0.01-0.55)
Motts Mill virus	Luteo-Sobemo	*D. melanogaster*	**1.27**(0.20-4.19)		
Galbut virus	Partiti-Picornabirna	*D. melanogaster*	**10.82**(7.09-16.91)	*D. suzukii*	**0.49**(<0.01-1.29)
DCV	Picorna-Calici	*D. melanogaster*	**2.57**(0.79-6.16)	*D. immigrans*	**0.13**(0.04-1.33)
Thika virus	Picorna-Calici	*D. melanogaster*	**0.64**(0.03-3.14)	*D. suzukii*	**0.06**(<0.01-0.54)
La Jolla virus	Picorna-Calici	*D. melanogaster*	**0**	*D. suzukii*	**0.23**(<0.01- 9.07)
Chaq virus	unknown	*D. melanogaster*	**14.27**(8.24-22.38)	*D. suzukii*	**0.35**(<0.01-1.08)
Prestney Burn virus	Luteo-Sobemo	*D. subobscura*	**56.8**(43.7-69.7)	*D. suzukii*	**0.03**(<0.01-0.57)
Cherry Gardens virus	Mono-Chu	*D. subobscura*	**2.08**(0.99-4.28)		
Kinkell virus	Picorna-Calici	*D. subsilvestris*	*unknown*	*D. suzukii*	**0.03**(<0.01- 4.84)
Teise virus	Luteo-Sobemo	*D. suzukii*	**15.31**(11.58-19.83)	*D. immigrans*	**0.03**(<0.01-1.16)
Medway virus	Luteo-Sobemo	*D. suzukii*	**0.06**(<0.01- 0.57)	*D. obscura*	**5.29**(0.49-19.34)
Snodland virus	Toti-Chryso	*D. suzukii*	**0.03**(<0.01-0.48)		
Larkfield virus	Toti-Chryso	*D. suzukii*	**0.03**(<0.01-0.48)	*D. subobscura*	**1.7**(0.67-3.86)
Beult virus	Hepe-Virga	*D. suzukii*	**1.02**(0.42-2.17)		
Eccles virus	Reo	*D. suzukii*	**9.52**(0.07-12.16)	*D. subobscura*	**38.04**(29.02-45.70)

D. suzukii was the only species to be surveyed from all three sampling periods in the UK (Fig. 3.1). The prevalence of infection by any virus was significantly different between 2014 and 2015 (LRT: 2ΔLogLik = 34.1, $p<0.001$) but not between 2015 and 2016 (LRT: 2ΔLogLik = 0.49, $p=0.48$). The most prevalent infection of *D. suzukii* in all UK samples was Teise virus, its prevalence also significantly increasing from 3.12% in 2014, to 24.9% in 2015 (LRT: 2ΔLogLik = 74.7, $p<0.001$) but the increase in prevalence to 27.8% in 2016 was not significant (LRT: 2ΔLogLik = 0.4, $p=0.51$). Teise virus was also detected in Japanese *D. suzukii* at a prevalence of 23.4% in 2016, significantly lower

than its prevalence in the UK in that year (LRT: 2ΔLogLik = 53.3, p<0.001). Muthill virus was the only virus to decrease significantly in prevalence over the course of three years in the UK, being detected at a significantly higher prevalence in 2014 compared to 2016 (LRT: 2ΔLogLik = 16.9, p<0.001).

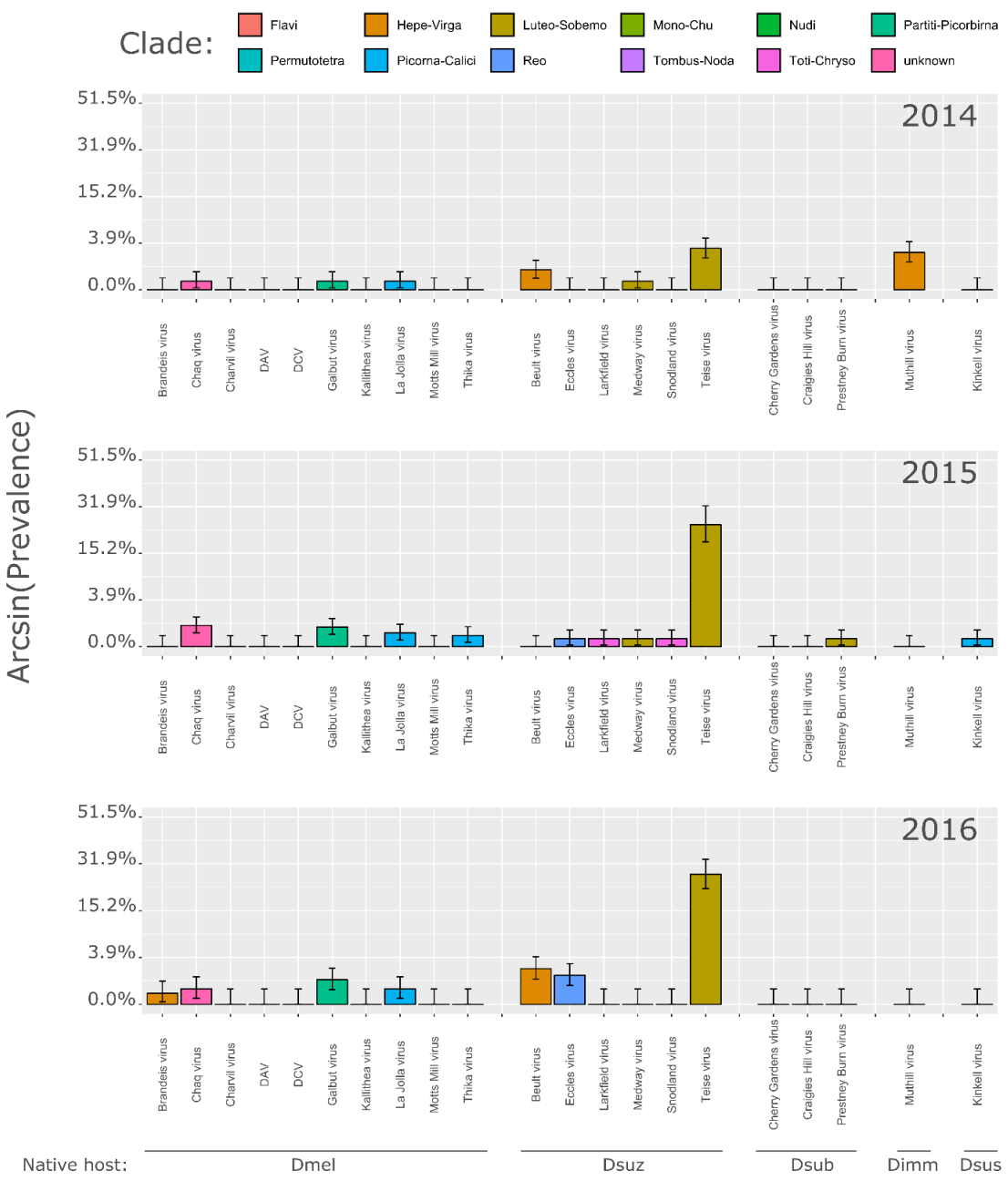

Fig. 3.1. The prevalence of 21 viruses in British *D. suzukii* sampled across three years. Error bars represent upper and lower bounds of a 2 log-likelihood interval. Bars are coloured by virus clade and grouped by native host.

Between the sampling years 2015 and 2016 there was a significant increase in prevalence of any infection in *D. immigrans* (LRT: 2ΔLogLik = 6.5, p<0.05) and *D. subobscura* (LRT: 2ΔLogLik = 19.4, p<0.001) but there was no significant difference in overall prevalence in any other species (Fig.3.2). Eccles virus prevalence in *D. subobscura* decreased significantly from 76.09% to 0% between 2015 and 2016 (LRT: 2ΔLogLik = 135.3, p<0.001).

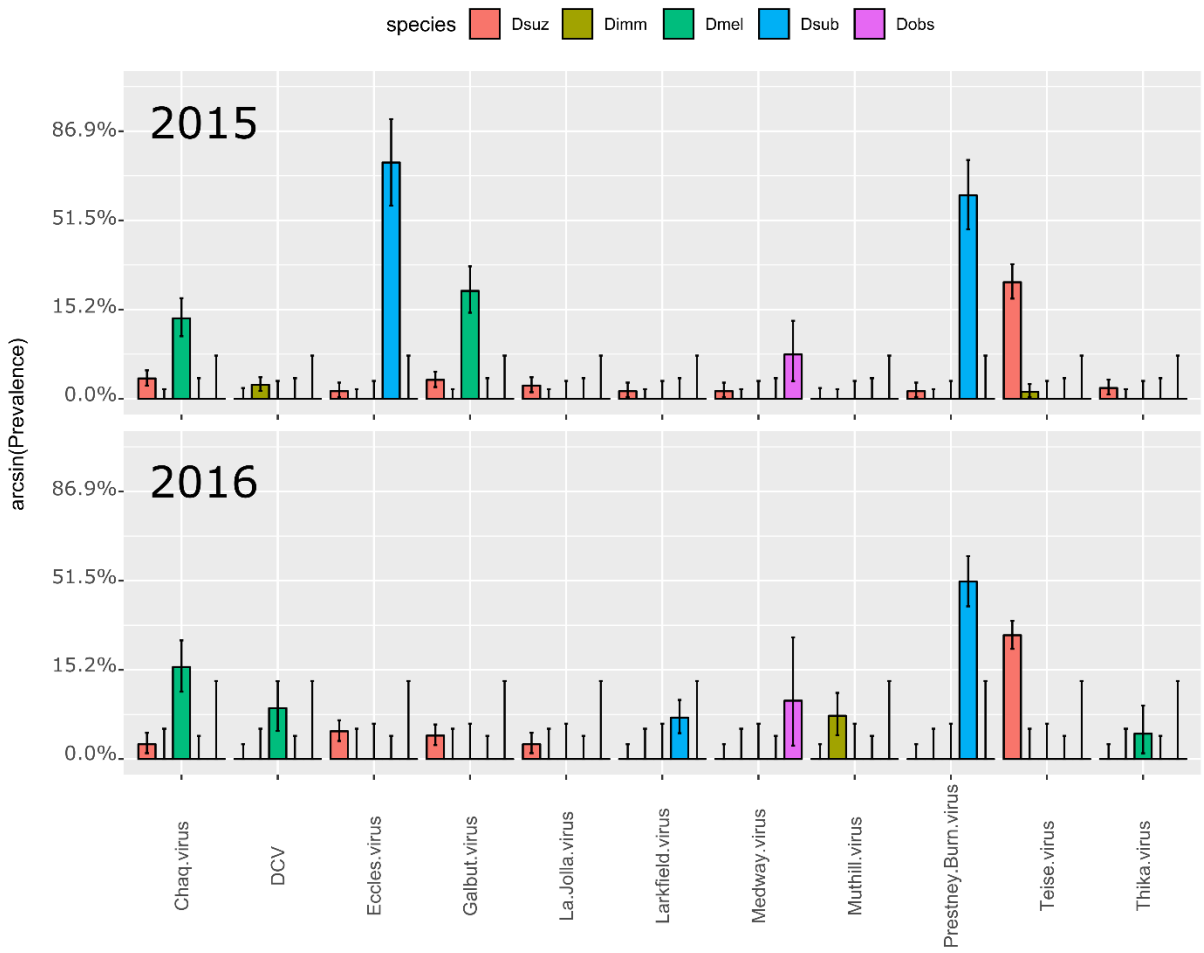

Fig. 3.2. The prevalence of 11 viruses detected in more than one host in British *Drosophila* sampled across two years 2015 & 2016. Error bars represent upper and lower bounds of a 2 log-likelihood interval. Bars are coloured by host species.

The two host species sampled in both the UK and Japan were *D. suzukii* and *D. immigrans* in 2016. The prevalence of all viruses are shown in figure 3.3. Muthill virus was the only virus in our study infecting both host species in both samples. The prevalence of Muthill virus was significantly higher in Japanese *D. suzukii* than in British flies of the same species in 2016 (LRT: 2ΔLogLik = 124.9, p<0.001). Muthill virus was detected at higher prevalence in British *D. immigrans* (3.72%) than in Japanese *D. immigrans* (0.64%): However, this difference was not significant (LRT: 2ΔLogLik = 3.3, p=0.07). In 2016 samples the diversity of viruses infecting *D. suzukii* was higher in the UK (7/21) than in Japan (4/21). Conversely, the prevalence of infection by any virus was higher in Japan (92.52%) than in the UK (31.73%). This difference between general infection prevalence was highly significant (LRT: 2ΔLogLik = 74.3, p<0.001).

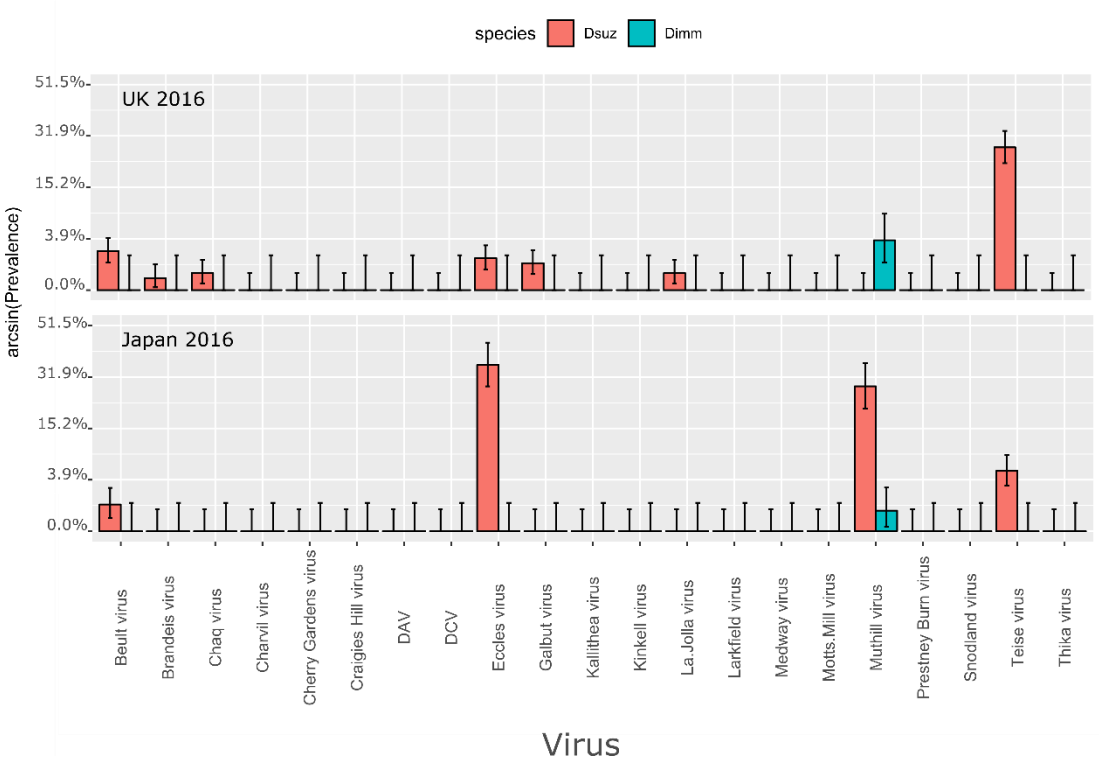

Fig. 3.3. The prevalence of 21 viruses in Japanese caught *Drosophila* sampled in 2016. Error bars represent upper and lower bounds of a 2 log-likelihood interval. Bars are coloured by host species.

Discussion

In this study we have examined the wild prevalence of 21 natural viral infections of five drosophila species. We found that prevalence of viruses infecting the invasive species *D. suzukii* changed significantly between years in its invaded range and was significantly higher in its native than in its invaded range. We also examined the host range of these viruses, finding that no virus infected more than two host species and tended to be significantly more prevalent in their assumed native host.

D. suzukii was first recorded in the East Malling Research, Kent in 2012 (Harris and Shaw, 2014), the same location from which all the UK samples in this study were collected. It is unlikely that populations of *D. suzukii* in this area are exclusively the decedents of a small invasive population seeded once in 2012. Firstly, active dispersal of flies from continental Europe, where this species was known to be established before 2012 (Calabria et al., 2012), may be possible, as it is for other fruit flies (Meats and Edgerton, 2008, Meats and Smallridge, 2007). Secondly, human mediated dispersal through the international fruit trade, may be continuously reintroducing individuals from distant, more established or even native populations (Fraimout et al., 2017). The influence of founder effects leading to a reduction in pathogen burden may, therefore, be diluted as newly introduced individuals reintroduce potentially 'escaped' pathogens from more established ranges (Colautti et al., 2004). Even given this possible dilution of the pathogen escape effect, we do find that British *D. suzukii* have an overall lower prevalence of virus infection than their native conspecifics during the same sampling year. This adds some credence to the theory of enemy

release: However, our findings should not be taken in isolation, as it is possible that a change in the type of virus infection experienced by invading *D. suzukii* has gone undetected in our assessment this subset of possible virus infections. Although viruses detected from a high abundance of reads in previous metagenomic surveys (see chapter 2) have been included, it is possible that one or more virus detected at low levels, but not surveyed for here, is highly prevalent.

Another factor limiting our ability to comprehensively test the temporal patterns of virus loss and gain in this invasive species is the lack of repeated years surveying within the native range. As we have seen from this study it is possible that the viruses of a particular species fluctuate significantly from year to year even in native species. Here, *D. subobscura*, native Western Europe (Ayala et al., 1989), had significant fluctuations in overall virus prevalence between sampling years. It is possible that such fluctuations are also seen between years in Japanese *D. suzukii* but through lack of temporal replication we are unable to detect them in this study. Additional sampling in the UK prior to the introduction of *D. suzukii* would also be illuminating in this context: the diversity of viruses in the studied populations of native flies being unknown before sampling started, two years after the first detection of *D. suzukii*. It is possible that even though *D. suzukii* is still considered invasive in the UK by most metrics, in terms of host-pathogen dynamics we may be late to observe the most dramatic virus host shifts. Understanding the time scales at which pathogens associated with an invasive species 'catch up' to the pioneering individuals at the edges of the invasive range, or at which pathogens are shared or gained with the native congeners is key to further study of the ecoimmunology of invasive species.

If parasites are lost during the course of an invasion event the consequences could be seen in the immune investment of invasive populations relative to their native conspecifics. Theory predicts that in a low pathogen environment, individuals who have divested away from immunity and into more invasive traits such as growth, reproduction or dispersal will predominate (Horrocks et al., 2011, Zuk and Stoehr, 2002). However, in the case that some pathogens in the invaded range are able to infect the newly arrived host, the most invasive individuals might not be those that invest away from defensive traits entirely, but those who possess a broad suite of protection against a diversity of potential threats. Studies simultaneously quantifying the three relevant aspects of pathogen burden, immune response and overall fitness are rare but critical to understanding the ecoimmunology of invasive species (Graham et al., 2011).

Classifying the viruses in this study according to the framework outlined by Fenton and Pedersen (2005), based on prevalence data alone, is somewhat speculative as we lack key transmission data for these viruses: However, we can make some inferences based on their wild prevalence alone. No viruses in this study appeared at consistently high prevalence's in multiple hosts, so from this data alone, none would appear to be truly multi-host pathogens or apparent multi-host pathogens. We would expect that viruses constituting spillover infections to be detected at low a prevalence in recipient host populations due to low transmission to this species from the endemic host. Furthermore we would expect such infections to remain at a low prevalence through time in recipient hosts due to low transmission between individuals of this species. Several of the viruses seen here to infect more than one

host, appear at consistently low frequencies in their secondary host, i.e. Galbut virus, DCV, Thika virus, La Jolla virus, Chaq virus, Prestney Burn virus and Teise virus. These viruses were not found to increase significantly in prevalence between years in their secondary hosts. Infections in the secondary hosts of these viruses may therefore represent spillover infections, lowly transmitted between individuals of the recipient species. The specific use of the term spillover, as defined above, does not preclude changes in infection prevalence of the secondary host, although lowly transmitted between recipient individuals and between endemic and recipient host populations, a true spillover virus could increase in prevalence as the reservoir species also increased its numbers. In the aforementioned example of West Nile Virus the changing prevalence of a single, relatively uncommon, avian species, the American robin (Turdus migratorius), appeared to be responsible for the majority of WNV-infectious mosquitoes and acted as the species equivalent of a super spreader for this multi-host pathogen (Marm Kilpatrick et al., 2006).

An emerging infection might be expected to show high prevalence in its secondary host as the transmission rate between individuals of that recipient species is, by definition, high. Eccles virus, first described from British *D. suzukii* (see chapter 2), may represent such an infection in *D. subobscura*. Its prevalence in its assumed endemic host, *D. suzukii*, was significantly higher in native Japanese populations than in invasive UK populations (Fig.3.3), possibly due to pathogen escape (see above), and although its prevalence significantly increased by 1.4% between 2015 and 2016, its prevalence remained relatively constant in the UK, only increasing by 1.5% between 2014 and 2016. In *D. subobscura*, its assumed secondary host, prevalence

was extremely high, in 2015 to 76.1%. Although prevalence of Eccles dropped significantly in 2016 it was not reported at all from British *D. subobscura* in 2011 (Webster et al., 2016). This spike in prevalence in 2015 could represent an epidemic outbreak of the virus in its secondary host. The pathology of this virus is unknown, however, it is closely related to a group of insect cypoviruses advocated for use as biological control agents (Zeddam et al., 2003a, Peng et al., 1998, Peng et al., 2000). This virus, belongs to the family Reoviridae, a group of viruses with a segmented dsRNA genome. It is possible that this multipartite genome, capable of reassortment, aids this type of virus in adaptation to new hosts (Worobey and Holmes, 1999). Reassortment has been linked to the emergence of other multipartite RNA viruses, including Influenza A viruses in humans (Webster et al., 1992, Li et al., 2004a, Garten et al., 2009) and Hantaviruses in a range of mammalian hosts. (Jonsson et al., 2010, Klempa, 2018).

4. Patterns of virus-induced gene expression in male and female *Drosophila melanogaster and D. suzukii*

This chapter will be submitted as a manuscript with the following additional authors:

William H Palmer, Darren Obbard.

William Palmer carried out injections of flies as outlined in the methods section.

Darren Obbard has provided feedback on parts of this manuscript and will continue to do so in preparation for submission.

Introduction

Viruses pose a constant immunological challenge to all living organisms, and hosts possess a suite of antiviral immune responses to fight infection and maintain fitness. In the insects, antiviral defences are best understood from studies of *Drosophila melanogaster* and this species has become an invaluable model for innate antiviral immunity (Hultmark, 1993, Mussabekova et al., 2017, Wang et al., 2010). These studies have implicated a variety of pathways in the antiviral defence of insects, including components of the RNA interference (RNAi), Toll, IMD, and Jak-STAT pathways. The templates of these innate defences are widely conserved across the tree of life (Hoffmann et al., 1999, Medzhitov et al., 1997, Arbouzova and Zeidler, 2006, Silverman and Maniatis, 2001, Zhang and Ghosh, 2001) with the innate immune

response thought to act immediately and instructively alongside the adaptive immune system of mammals (Fearon and Locksley, 1996, Hoffmann et al., 1999).

Of these pathways, the best-studied and arguably most important for invertebrate viral defence is the RNAi pathway (Bronkhorst and van Rij, 2014, Zambon et al., 2006, Merkling and van Rij, 2013, Obbard et al., 2009a). This pathway requires the uptake of viral dsRNA (Saleh et al., 2009), which is then recognised and 'diced' into 21-nt short interfering RNAs (siRNA) by the endonuclease Dicer-2 (Ding and Voinnet, 2007). The resulting siRNAs are then bound by Argonaute-2 and guide the RNA induced silencing complex (RISC) by sequence complementarity to other viral RNAs for destruction. Very recently an additional systemic memory element to this response has been reported (Tassetto et al., 2017, Saleh et al., 2009, Attarzadeh-Yazdi et al., 2009, Poirier et al., 2018), in which haemocytes endogenise fragments of RNA virus as DNA copies, and these endogenous copies form a source of secondary viral siRNAs.

The Jak-STAT pathway, which is responsible for the expression of several immune related proteins and the promotion of cellular immune responses (Sorrentino et al., 2004), is also known to be required for antiviral defence in *D. melanogaster* (Dostert et al., 2005, Huang et al., 2013). Interestingly, a ligand activating the Jak-STAT pathway, vago, is dependent on Dicer-2 for expression, providing a possible interaction between the RNAi and Jak-STAT pathways (Deddouche et al., 2008, Paradkar et al., 2012). Other components of the *Drosophila* antiviral immune response include two Nf-κB signalling pathways that also mediate the expression of antimicrobial peptides (AMPs), are the Toll and Immune deficiency (Imd) pathways.

Despite their primary association with antifungal and antibacterial defence, studies in *Drosophila,* and *Anopheles* mosquitos, have shown a role for these pathways in the antiviral response (Costa et al., 2009, Zambon et al., 2005, Avadhanula et al., 2009). A mechanism independent of these pathways, Toll-7 activated autophagy, has also proposed as a constituent part of the antiviral response in *Drosophila* (Nakamoto et al., 2012, Shelly et al., 2009).

Transcriptome-wide expression analysis, originally using microarrays and more recently RNA sequencing, suggest that components of all of these pathways can be upregulated in response to viral challenge. For example, infection of *D. melanogaster* with Drosophila X virus (DXV: a double-stranded RNA Birnavirus that persistently infects some *Drosophila* cell cultures) is reported to elicit increased expression of AMP genes, dependent on the activation of Toll and/or Imd pathways, to similar levels as seen in bacterial infection (Zambon et al., 2005). Similarly, genes for the AMPs Diptericin, Defensin, and Drosomycin, regulated by the Toll and Imd pathways, were also upregulated in transgenic flies with inserted replicon from Sindbis virus (SINV), a positive-sense ssRNA alphavirus of mosquitoes (Avadhanula et al., 2009). However, infection with cricket paralysis virus (CrPV), a positive sense ssRNA Cripavirus, did not induce any alteration of IMD/Toll mediated AMP expression at the same time point (Costa et al., 2009) and infection with *D. melanogaster* Sigmavirus (DmelSV, a vertically transmitted -ssRNA Rhabdovirus) gave conflicting results: Carpenter *et al.* (2009) found no change in expression of immune related genes in the Imd, Toll or Jak-STAT pathway, contrasting with Tsai et al. (2008) who found up-regulation of a selection of AMP and upstream regulator genes. Expression analyses

therefore imply that the response of the Toll and Imd pathways, and the resulting expression of AMPs, may depend heavily on the type of infecting virus. The same is true of the Jak-STAT pathway, with *Drosophila* C virus (DCV) and Flock House virus (FHV) both inducing an up-regulation of the Jak-STAT induced *vir-1* gene (Dostert et al., 2005, Hedges and Johnson, 2008), in contrast to DMelSV which elicits no such response (Carpenter et al., 2009, Tsai et al., 2008). The virus specific nature of this pathway is further supported by Kemp et al. (2013) who showed flies mutant for JAK are more susceptible to infection by DCV and CrPV but not SINV, DXV, invertebrate iridescent virus type 6 (IIV-6) or vesicular stomatitis virus (VSV). It is important to bear in mind when considering the results of these antiviral expression studies that particular pathways and components may be responding to different elements of the infection cycle: some regulation relating directly to viral replication, the RNAi pathway for example working in the presence viral RNA (*); others being associated with the damage caused by the infection, for example IMD and Jack/STAT pathways associated with bacterial defence in the gut (*) may be responding to bacteria leaking into tissue from the gut after gut lining is damaged by viral infection (*). This effect may be especially prominent in flies infected orally, depending on the tropism of that particular virus. Changes in expression of host genes in response to infection are not necessarily the result of host immune defence, and are often modulated by the virus to its own advantage. Viruses display a raft of mechanisms for manipulating their environment (the host). This can be to the end of evading the host's defensive response or manipulating the cellular environment to better suit replication, for example through pro-viral metabolic changes (Mazzon et al., 2018, Fontaine et al.,

2015, Yogev et al., 2014, Thai et al., 2014). An example of counter defensive manipulation can be seen in many viruses that suppress the antiviral RNAi pathway. Viral suppressors of RNAi (VSRs) have been described from a diverse range of mammalian (Bennasser et al., 2005, Andersson et al., 2005, Haasnoot et al., 2007, Li et al., 2004b, Wang et al., 2006b) and plant viruses (reviewed in; Roth et al. 2004). In *Drosophila*, virus-induced immune suppression is known from several viruses which encode proteins targeting key steps in the RNAi response (Li and Ding, 2006). DCV (van Rij et al., 2006), FHV (Li et al., 2002, Wang et al., 2006a), and CrPV (Wang et al., 2006a) all encode for VSR's essential for successful infection.

Although the response of *D. melanogaster* to immune challenge has been well studied, there have been surprisingly few studies of the immune response of other *Drosophila*. This is significant because, while it is reasonable to think that most components of the *Drosophila* immune system will be shared with close relatives, genes related to immune function tend to have a rapid rate of evolution compared to other genes (Sackton et al., 2007, Obbard et al., 2006, Obbard et al., 2009b). Furthermore, although the underlying sequences involved in immune function are observed to have a faster rate of evolution, how this genomic change between species corresponds to changes in immune expression is still poorly understood (although see; Salazar-Jaramillo *et al.*, 2014). While genome sequencing has identified a suit of immune genes across the genus *Drosophila* (Ekengren and Hultmark, 2001), the identification of novel immune system components in other *Drosophila* relies heavily on homology to known *D. melanogaster* genes, limiting the scope of discovery to genes paralogous to those in *D. melanogaster*. Transcriptome

studies allow the identification of novel immune related genes that share little or no homology to those in well studied relatives. Despite this, comparative studies of the transcriptional response between different insect species are relatively rare. The response of two *Drosophila* species, *D. melanogaster and D. virilis*, has, however, been compared for both bacterial (Sackton and Clark, 2009) and fungal (Seto and Tamura, 2013) infections. Sackton and Clark (2009) found substantial differences in the induction of AMPs between the two species: *D. virilis* showing comparatively strong induction of Diptericin encoding genes in particular. A result partially mirrored under fungal infection, where Diptericin was found to be the most highly expressed AMP in *D. virilis* larvae infected with *Penicillium* fungi (Seto and Tamura, 2013).

Similarly, despite phenotypic differences in male and female survival after infection observed in many animals, including *Drosophila* (Taylor and Kimbrell, 2007) and a wealth of theoretical explanations for the patterns observed (Zuk and McKean, 1996, Marriott and Huet-Hudson, 2006, Rolff, 2002, Klein and Flanagan, 2016), there have been surprisingly few systematic studies of the transcriptional immune response underlying this dimorphism. Sex specific differences in survivorship are not always observed in the same direction and predicting the effects of sex on immune function are complicated by interactions with diet (McKean et al., 2005), age (Kubiak and Tinsley, 2017), and levels of sexual activity (McKean and Nunney, 2001, Schwenke et al., 2016). Investigating the genetic basis of this difference, Hill-Burns and Clark (2009) quantified variation in *D. melanogaster* immune phenotypes as a function of polymorphisms in X-linked immune genes, including Toll and Imd pathway components. They found significant dimorphic effects on the association between

immunocompetence and genotype. However, this dimorphic effect was not apparent when assessing the autosomal genetic basis for variation in immune function (Lazzaro et al., 2004) suggesting that sex chromosomes are a key determinant of sexual dimorphism in the *Drosophila* immune response. The Toll pathway is further implicated as factor in sexual dimorphism in immune response to bacteria by Duneau et al. (2017) who found the pathway to be dimorphic in genome-wide gene expression and in induced response to infection.

In this study we use full transcriptome sequencing to compare the transcriptional response of male and female *D. melanogaster* to infection by a natural +ssRNA virus (DCV) and Kallithea virus (KV) a natural dsDNA virus of this species (Webster et al., 2015). We examine male and females separately to test for for potential sex specific differences in immune response (Ranz et al., 2003, Rolff et al., 2005). We then go on to compare the antiviral response of the invasive pest *Drosophila suzukii*, to that of *D. melanogaster*, again examining the difference between males and females. The immune response of *D. suzukii* is of particular interest because is an invasive pest of soft fruit, native to SE Asia that belongs to the *Melanogaste*r species group but that displays a markedly different ecology to *D. melanogaster.* Females possess a serrated oviscapt, allowing them to oviposit under the skin of ripening fruit still on the tree, where larvae develop in a microbial environment different to that of their saprophagous relatives (Chandler et al., 2014, Hamby et al., 2012).

Methods

Sample preparation for RNA-sequencing

To understand how the transcriptional response to viral challenge varies between host species and sexes, and between DNA and RNA viruses, we used a Nanoject II (Drummond Scientific) to abdominally inject 60 male and 60 female adults in vials of 10, of both *D. melanogaster* (Oregon R) and *D. suzukii* ('Davis', isofemale line sequenced by Chui et al. 2009) with isolates of DCV or KV. We raised flies on standard corn starch *Drosophila* medium (Lewis, 1960) at a 12:12 LD cycle at 24°C. All flies were mated and between 7-14 days old at the time of injection. We obtained cultures of KV through isolation from wild flies outlined in detail by (Palmer et al., 2018b). Briefly, wild flies testing positive for KV were homogenised and serially passaged through mutant Dicer$^{-2L811fsX}$ flies, which lack a robust antiviral immune response (Lee et al., 2004). After three passages, homogenate was cleared by centrifugation (max 6000 x g) and filtering through a Millex 0.45 µm polyvinylidene fluoride syringe filter. This crude virus solution was then ultra-centrifuged to separate KV from other viruses using equilibrium buoyant density centrifugation in iodixanol ("OptiPrep", Sigma-Aldrich). Infectious dose (ID50) was calculated by injection into Oregon R *D. melanogaster* and analysing viral titre by qPCR after 5 DPI. Aliquots of 10^5 ID50 were stored at -80°C until use in this experiment. Simultaneously uninfected Dicer$^{-2L811fsX}$ were homogenised, centrifuged in the same way and equivalent fractions of the gradient solution extracted to act as sterile control solution. We obtained DCV isolates courtesy of the Jiggins lab, University of Cambridge which were produced by infecting Schneider Drosophila line 2 (DL2) cells, cultured at 26.5°C in Schneider's

Drosophila Medium (Invitrogen). Infected cells were then filtered through 0.45 µm and centrifuged at 13500 rpm for ten minutes to remove cellular debris and bacteria. Aliquots of a 10^{-4} dilution of the virus suspension were prepared using 50 mM TE buffer and frozen at -80°C (Longdon et al., 2012).

Three days post infection (DPI) we homogenised flies in Trizol® (Ambion) solution and extracted total RNA according to the proprietary protocol. To enrich these samples for mRNA, we treated with Turbo DNase (Invitrogen) and poly-A selected libraries using NEBNExt® Ploy(A) mRNA Magnetic Isolation Module (NEB) before preparation of strand-specific paired-end libraries using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB). Libraries were then pooled and sequenced by Edinburgh Genomics over three lanes of an Illumina HiSeq 4000 platform with strand-specific 75 nucleotide paired end reads. Although not previously detectable by PCR, we subsequently identified a low level of Drosophila A Virus (DAV) contamination in both KV treated and untreated flies, reflecting the widespread occurrence of this virus in fly stocks and cell cultures. We submitted all reads to the European Nucleotide Archive under project accession ERP023609.

Differential expression analysis

Paired end reads were trimmed for primer and adapter sequences using cutadapt (V1.8.1; Martin, 2011) before mapping to either *D. melanogaster* (FlyBase release r6.15) or *D. suzukii* (Dsuzukii.v01) genomes, and all known *Drosophila* virus genomes using STAR (V2.5.3a; Dobin et al, 2013). We set a maximum intron size of 100 KB, but otherwise used default settings. We used the 'featurecounts' command in the Subread package (V1.5.2; Liao et al, 2013) to count the number of reads mapping to

each gene and used these raw count data for differential expression analysis using DESeq2 (V1.16.0; Love et al, 2014). DESeq2 fits a generalised linear model for each gene, where read counts are modelled as a negative binomially distributed variable (Anders and Huber, 2010, Love et al., 2014). The DESeq2 model includes a normalisation step consisting of a median of ratios method, which accounts for sequencing depth and RNA composition (Love et al., 2014, Anders and Huber, 2010). Our design matrix included sex, virus infection status, species and corresponding interactions, allowing us to test for expression changes following virus infection and how these changes differ both between sexes and host species. To account for the unintended presence of DAV, and differences in the level of DAV within and between the treatments, we also included DAV titre as a continuous predictor in our models. We calculated $\log_2$ fold changes in DESeq2, testing for significance using Wald tests and performing principal component analysis using the 'plotPCA' function included in DESeq2 (Love et al., 2014). Genes reported as significantly differential expressed are those with an adjusted *p* value (Benjamini–Hochberg correction) of <0.01 and a fold change of >2 (log2fold change > 1). Strict significance and fold change thresholds were deliberately enforced to limit the analysis to those genes most likely to have a biological effect specific to treatment. Comparisons of normalised read counts between groups were analysed with Dunn (1964) Kruskal-Wallis test for multiple comparisons.

Comparison of response between these two host species was only possible for *D. suzukii* genes with direct one-to-one orthologues in *D. melanogaster*. Homology information was provided by Dr Joanna Chui of UC, Davis and

Spottedwingflybase.org. 9954 Genes showed direct homology between the two species and it is with this subset of shared genes that the comparison between the two species is made.

Results

A comparison of the response of *D. melanogaster* to RNA and DNA viruses

To compare the differences in antiviral response to two highly divergent viruses we examined the whole genome virus-induced expression patterns of *D. melanogaster*. KV was injected into *D. suzukii* but sequencing returned very low numbers of reads mapping to any part of the KV genome, suggesting that the virus had not undergone active replication and the treatment had failed to initiate an active infection. Libraries prepared from *D. melanogaster* generated 496,754,883 reads that mapped to the fly genome and 83,323,999 that mapped to *Drosophila* viruses, of which 81,661,200 mapped to DCV and 1,629,934 mapped to KV. Of the remaining virus-mapped reads 22,526 mapped to the aforementioned contaminant DAV. Counts of reads mapping to known components of the virus genomes confirmed that DCV and KV both replicated to high titres in *D. melanogaster* (Fig.C.1). Normalised counts of a representative KV gene, an ORF similar to DNA-PolB (KX130344), were significantly higher in flies treated with KV than in untreated controls (Males: $z= -2.69981672$, $p <0.01$; Females: $z= -3.38854548$, $p <0.001$) or DCV-treated flies (Males: $z= -3.18192685$, $p <0.01$; Females: $z= -2.63094384$, $p<0.05$). Similarly, normalised counts

for DCV were significantly higher in DCV-treated flies than in KV-treated (Males: z= 2.4933846, p <0.05; Females: z= 3.7811767, p<0.001) or control treated flies (Males: z= -2.3563855, p <0.05; Females: z= -3.2057803, p<0.01).

A total of 556 *D. melanogaster* genes were significantly differentially expressed under challenge by either virus. Similar numbers of genes were detected to be DE in KV treatments (313) and in DCV treatments (366) although less than half of these (123) were differentially expressed in both treatments (Fig 4.1.). Of these shared genes, 69 are unnamed 'computed genes' (CG) with no currently characterised function. 238 genes showed significant differentially expressed under KV treatment in females, 59 of which were expressed at a significantly higher level in KV than control treatments. Of these genes two have previously been implicated in anti-viral immune response:

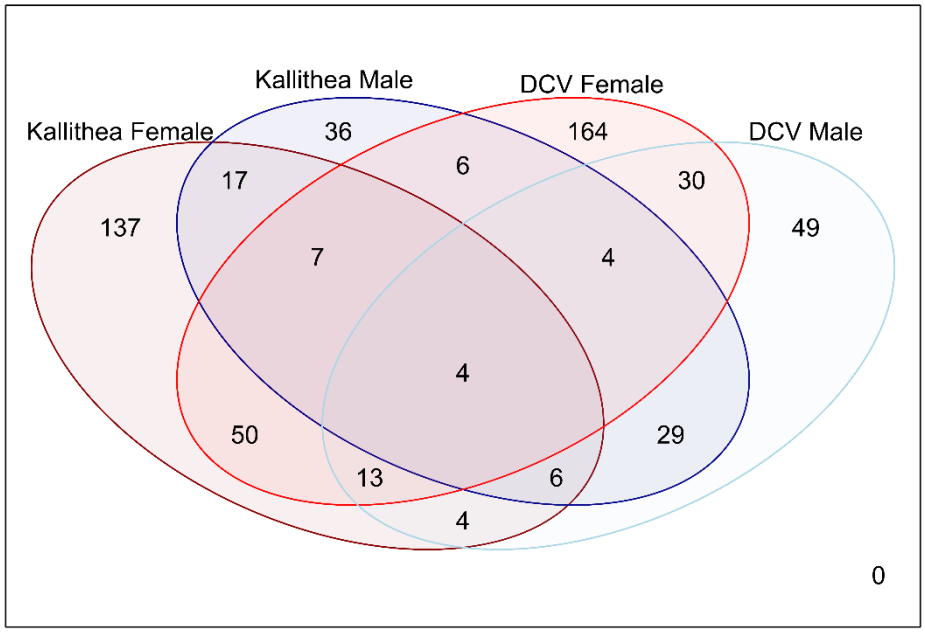

Fig. 4.1. The number of significantly differentially expressed genes that are common or unique to infection by DCV or KV in male or female *D. melanogaster*.

Vago, a gene associated with the antiviral RNAi pathway (Deddouche et al., 2008) was significantly up regulated in KV treated females (Log2fold change: 1.69, padj: <0.001) but not in males (Log2fold change: 0.58, padj=0.14). Ref(2)p was also up regulated in KV infected flies: females (Log2fold change: 1.11, padj: <0.001) and males (Log2fold change: 1.26, padj: <0.001). Ref(2)p, which had the highest levels of significance of any differentially expressed gene in male KV-treated flies ($p=7.96 \times 10^{-14}$), is required by the Toll immune response and has shown to be involved with controlling Sigma virus replication in *Drosophila* (Avila et al., 2002, Contamine et al., 1989). KV infection induced significant up regulation of AttD, an AMP, in females (Log2fold change: 2.69, padj: <0.01) but not to a significant degree in males (Log2fold change: 2.22, padj: 0.09).

Of the 366 genes differentially expressed in DCV-treated flies 159 and 70 genes increased in expression by more than two fold over controls in females and males, respectively. *Upd2* and *Upd3* were among these genes highly expressed in both females (Log2fold change: 6.68, padj: <0.001 and Log2fold change: 3.23, padj: <0.001, respectively) and males (Log2fold change: 2.79, padj: <0.001 and Log2fold change: 1.65, padj: 0.0019, respectively). Proteins coded for by Upd2 and Upd3 have been shown to induce the Jak-STAT-dependent activation of *tot*A in the *Drosophila* fat body (Hombría et al., 2005, Agaisse et al., 2003). Also significantly differentially expressed in DCV-treated females (Log2fold change: 2.72, padj: <0.001) and males (Log2fold change: 1.84, padj: <0.001), *Socs36E*, transcription of which is Jak-STAT mediated (Karsten et al., 2002), encoding for a protein shown to negatively regulate Jak-STAT signalling *in vivo* (Stec et al., 2013). The AMP encoding gene Attacin-D (AttD) was also

significantly induced under infection by DCV in males (Log2fold change: 2.43, padj: <0.01) and females (Log2fold change: 3.29, padj: <0.01). Another AMP gene Drosomycin-like 3 (Drsl3) was significantly up regulated in both males (Log2fold change: 3.05, padj: <0.01) and females (Log2fold change: 2.84, padj: <0.01) under DCV treatment but not in KV treatments.

Only four genes were significantly differentially expressed under infection by either virus in both sexes: *Jonah 65Ai*, CG32368, CG33926, and CG43064. Although the exact molecular function of the three CG genes is unknown, CG33926 has been recorded as being very highly induced in *Drosophila* larvae under Sindbis virus infection (Brown et al., 2014). The serine protease gene *Jonah 65Ai* (Jon65Ai) was significantly down regulated in all *D. melanogaster* virus treatments, a pattern concurrent with previous studies of DCV (Chtarbanova et al., 2014) and Sigma virus infection (Carpenter et al., 2009).

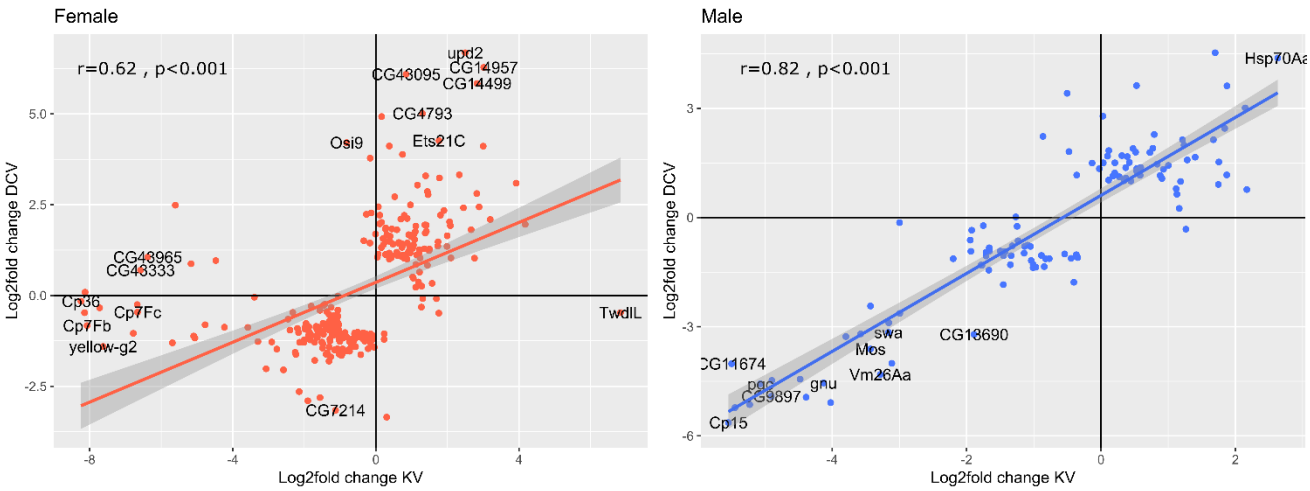

Fig. 4.2. The correlation between differentially expressed genes in each virus infection separated by sex.

To understand the overall similarity in response of *D. melanogaster* to different viral challenges we compared the expression of the genes showing significant differential expression over the control under each treatment, to those of the other the virus treatment type (Fig. 4.2). The response of male flies to DCV and KV virus were very highly correlated (Spearmans rank correlation, r= 0.82, n= 119, p<0.001) while responses in female flies, although still significantly positively correlated (r=0.62, n=556, p<0.001) included a number of genes highly down regulated under KV infection and unchanged under DCV infection. Of the 10 genes most highly down regulated (<-5.5 Log2fold change) in females treated with KV four are Chorion proteins (Cp36, Cp38, Cp7Fb, and Cp7Fc) involved in eggshell assembly. This finding concurs with the discovery that KV infection induces a reduction in mature ovaries and increased apoptosis in the ovaries of female *D. melanogaster* (Palmer et al., 2018b). The gene most highly expressed in KV treated females, TwdlL (l2fc: 6.84, padj: <0.001) shows no significant differentially expressed in DCV treated females (l2fc: -0.47, padj: 0.88) or any other treatment.

A comparison of the response of *D. melanogaster* and *D. suzukii* to DCV infection

To understand how the response to viral challenge of two closely related hosts compares, we measured transcriptional response to infection by DCV in both *D. suzukii* and *D. melanogaster*. Counts associated with the genome of DCV show that the virus grew to much higher titres in *D. melanogaster* than in *D. suzukii*, nevertheless, more DCV was present in treated *D. suzukii* than in controls of either

species (Fig. C.1) suggesting that the virus had undergone at least some active replication in the non-natural host.

The transcriptomic response of *D. suzukii* to DCV infection was muted in comparison to *D. melanogaster:* 25 genes significantly differentially expressed in either sex compared to 258 in *D. melanogaster*. All 25 genes were genes significantly differentially expressed in females, 24 of which were significantly up regulated in response to DCV infection. Of these genes there was no enrichment for any particular GO term and only one gene, for the AMP Defensin (Def), has any known immune function (GO:0002376) in *Drosophila* although this association is with defence against gram positive bacteria (Imler and Bulet, 2005). It was significantly up regulated in female *D. suzukii* (Log2fold change: 4.42, padj: <0.001). The one gene which decreased in expression compared to the control was Cyp12c1 belonging to the cytochrome P450 family (Log2fold change: -1.01, padj: 0.0083).

Expression patterns of the 281 genes significantly differentially expressed in either sex of either species were weakly correlated between the two host species (Fig. 4.3). In males expression of these genes were very weakly positively correlated (r_s=0.19, p= 0.013) with all genes significantly upregulated in *D. melanogaster* showing no significant change in expression in *D. suzukii*. The correlation between female responses was stronger (r_s = 0.31, p<0.001) but with many of the genes positively differentially expressed in *D. melanogaster* showing weakly significant change in *D.*

suzukii. For example, Upd2 and Upd3 which were strongly expressed in *D. melanogaster* not meeting significance thresholds in *D. suzukii*.

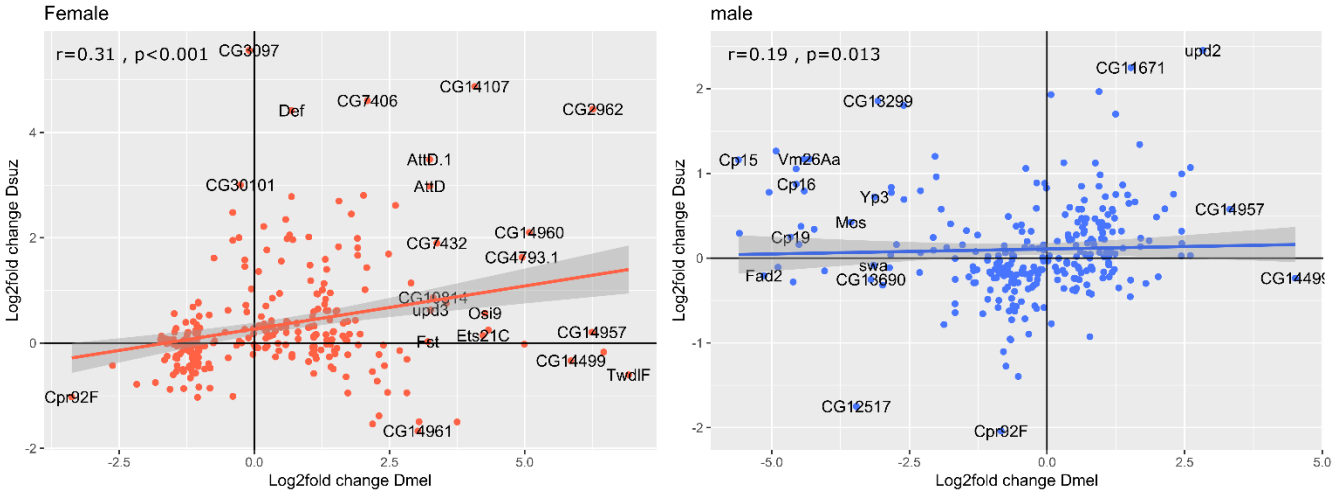

Fig. 4.3. The correlation between differentially expressed genes in *D. suzukii* or *D. melanogaster* under infection by DCV separated by sex.

A comparison of the responses of males and females to virus infection

Principle component analysis of normalised read counts from both species showed that 97% of the overall variance can be explained by two principle components: PC1, 54%, separating the sexes of each species and PC2, 43%, separating treatments based on species (Fig. 4.4).

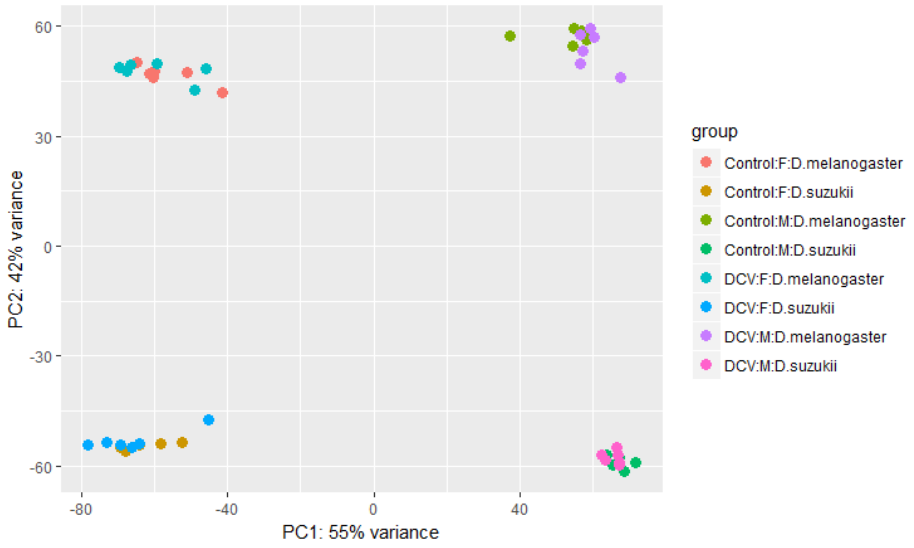

Fig. 4.4. Principle component analysis of variation in normalised read counts for counts mapping to genes with one-to-one orthologues between *D. suzukii* and *D. melanogaster*.

Males and females of both species can be compared for their responses to infection by DCV virus: by taking the 281 genes differentially expressed in both species and comparing their expression patterns in each sex. For this analysis we removed any genes that are known to show sex specific expression, for example those expressed only in ovaries or testis, in *D. melanogaster*. This removed 37 of the 281 genes differentially expressed in either species. The expression patterns of these genes were significantly positively correlated between sexes in both species (Fig. 4.5). In *D. melanogaster* the positive correlation was much stronger (r_s= 0.68, p<0.001) than in

D. suzukii (r_s= 0.39, p<0.001). In KV-treated *D. melanogaster* male and female responses were less well correlated with each other (r_s= 0.49, p<0.001) than in DCV.

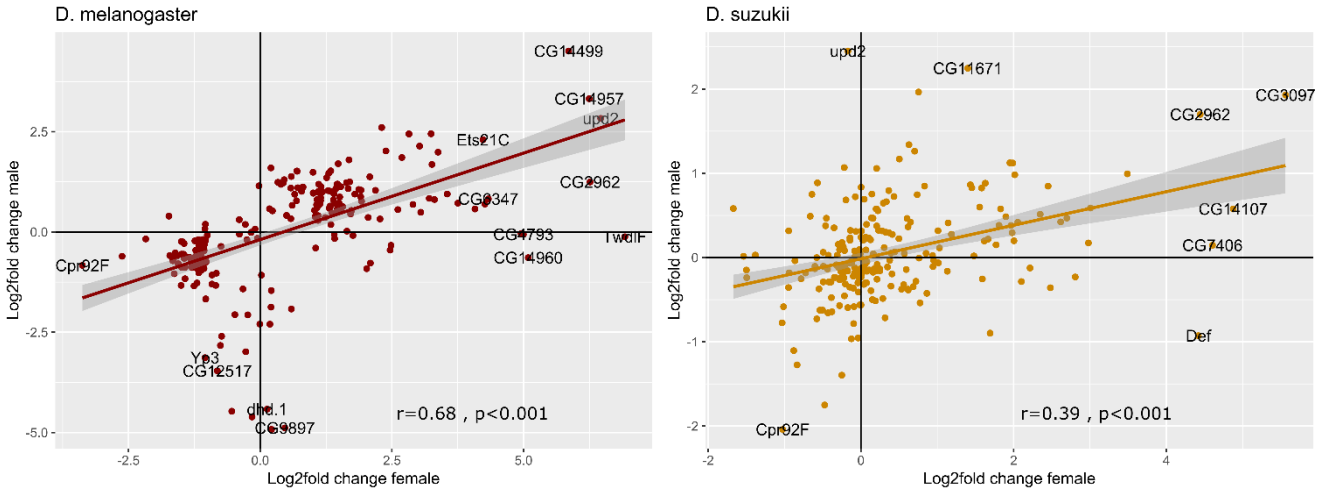

Fig. 4.5 The correlation between differentially expressed genes in males and females under infection by DCV separated by species.

Discussion

In this study we have highlighted some broad similarities and differences between the virus-induced transcriptional responses of *D. melanogaster* to two types of viral threat and between two different host species, *D. melanogaster* and *D. suzukii*, to infection by the RNA virus DCV. The similarity of expression profile between male and female also differs between species and type of infection.

The two viruses used in this study represent distant stands of the virus phylogeny: DNA and –ssRNA viruses being separated by potentially millions of years of coevolution with their host (McGeoch et al., 2000, Nasir and Caetano-Anollés, 2015).

It is maybe no surprise, then, that these highly divergent viruses elicit somewhat distinct responses in their natural host. Large DNA viruses such as nudiviruses have genomes many times the size of simple RNA viruses such as DCV. They have the capacity to encode many more proteins, some of which potentially combatting their host's immune response, for example immune suppressors discovered in the Baculoviridae (Mehrabadi et al., 2015) or Poxviridae (Haga and Bowie, 2005). Kallithea virus itself is now known to encode a suppressor of the Toll (Palmer et al., 2018a) suggesting some role for this pathway in the hosts immune response to this type of viral infection. In this study we see increased expression of the Toll pathway component Ref(2)P in KV-treated flies of both sexes. Ref(2)P is required for the activation of the Toll signalling pathway acting down-steam of the nuclear translocation of *Dif* (Avila et al., 2002). Primarily associated with antifungal immune response through the production of AMPs, it is unclear the exact mechanism by which Toll signalling initiates antiviral response. In line with previous studies into the anti-RNA virus response of Drosophila we detect the upregulation of the various components of the Jak-STAT pathway in *D. melanogaster* infected by DCV. Up2, Up3 and Socs36E, were all significantly up regulated in male and female *D. melanogaster*.

Further work would be needed to establish the generality of the differences seen here between RNA and DNA virus infections. Certain elements of the *Drosophila* immune defence seem to be virus specific, with a range of transcriptional responses recorded from different a number of different RNA viruses (Kemp et al., 2013, Hedges and Johnson, 2008, Carpenter et al., 2009). Our knowledge of anti-DNA virus immunity in Drosophila is, however, limited. Studies to date have used Invertebrate

iridescent virus 6 (IIV-6), a moth virus, as a model to study the DNA virus specific responses to infection (Bronkhorst et al., 2012, Bronkhorst et al., 2014). These studies show a role for the known anti-RNA virus RNAi pathway, with mutants for this pathway having modest increases in viral titre. However, much of the anti-DNA virus response remains to be characterised. It seems probable that there are DNA virus specific elements to anti-viral response in Drosophila yet to be described. This may be reflected in the high numbers of genes seen upregulated in this study with poor or no functional annotation. "CG" genes are among some of the most strongly differentially expressed genes in KV-treated flies some of which may constitute components of undescribed anti-DNA virus pathways. Characterisation of these genes, strongly differentially expressed under KV treatment, could be achieved through a number of different functional annotation techniques (Pellegrini et al., 1999). We have attempted here to analyse not only the virus-induced responses of the natural host of the RNA virus, DCV, but also to assess the response of a non-native host in *D. suzukii*. The response of *D. suzukii* was severely muted in comparison to *D. melanogaster* and this could be for a number of reasons. Firstly, DCV is not a natural infection of *D. suzukii* (see chapter 3). It has shown to replicate in *D. suzukii* (Cattel et al., 2016) and cause a moderate level of pathology comparable to that caused in its natural host (Lee and Vilcinskas, 2017, Gupta et al., 2017). However, at the time point flies were homogenised in this study viral titres were much lower in *D. suzukii* than the natural host *D. melanogaster*. This one measurement of viral titre reflects only a snapshot of the entire infection cycle. It is possible that at this time post-infection DCV was at different point in its infection cycle in each host: replicating more slowly

in the non-natural host, *D. suzukii*. This time point was picked as previous work has shown three DPI to be the peak in viral titre for DCV infection in *D. suzukii* (Lee and Vilcinskas, 2017) and *D. melanogaster* (Gupta et al., 2017). To confirm whether the genes shown here to be implicated in virus-induced response in *D. melanogaster* also changed in expression at some point during the infection cycle of DCV in *D. suzukii*, one could analyse the expression levels of specific genes through the course of time, for example by RTqPCR.

In this study we see a substancial difference in the transcriptional response of male and female flies to infection. The difference between sexes contributing as much to the overall variance in response of individuals as the difference between species even with sex specific, including X-linked, genes removed from the analysis. Differences in in infection phenotype have been reported in insect-virus systems (*). In our specific case, DCV infection is known to have significantly different effect on the behaviour of male and female *D. melanogaster*, with females showing reduced levels of locomotor activity compared to healthy flies and effect not mirrored in male flies (Vale & Jardine, 2015*; Gupta et. al. 2017). This may be due to increased energy expenditure in female flies, burdened with the expensive process of producing protein rich eggs they may be more susceptible to the energetic cost of infection. These dimorphisms are no doubt underpinned by measurable differences in gene expression between the sexes but linking differences in infection phenotype to differences in expression profiles is not straight forward. In some cases, a causative association can be made between sex specific pathology and gene expression: for example, in this study we find the known pathology of Kalithea virus to reduce egg production and cause

ovarian apoptosis (*) concurrent with a reduced expression of Chorion protein genes essential to egg production. Interestingly the magnitude of dimorphism seems to vary between the two closely related species in our study: females of the two species studied being positively correlated to each other and males being uncorrelated for our specified set of genes. Further molecular level studies of our less well studied species, *D. suzukii*, would be needed to unpick the mechanistic causes of this disparity.

5. General discussion

Summary of the field

A decade ago *Drosophila suzukii* was an infrequently-considered Asian Drosophilid with virtually no status as pest and of almost no interest to the scientific community. However, in the last ten years *D. suzukii* has transcended from being innocuous to notorious: It has now come to the attention of a number of scientific fields and invaded many of the horticultural variety. The financial damage it has caused as a pest has probably been under reported but the few studies that estimate its cost to the soft fruit industry predict the numbers to be in the hundreds of millions, even for specific growing regions (Farnsworth et al., 2017, Goodhue et al., 2011, De Ros et al., 2015). Along with the potentially devastating effects on individual fruit crops (Walsh et al., 2011) the rapid spread of this fly across the globe (Fraimout et al., 2017) make it one of the most serious invertebrate pests in modern horticulture.

This status has brought *D. suzukii* to the attention of scientists in its newly invaded ranges as well as in its native SE Asia. Primarily, and understandably, the research undertaken has had the aim of finding control solutions (Schetelig et al., 2018). Advancements have been made in the form of product screening for effective chemical pesticides (Cuthbertson et al., 2014a, Swoboda-Bhattarai and Burrack, 2018, Cahenzli et al., 2018) as well as more integrated solutions such as biological control (Girod et al., 2018, Wang et al., 2018, Gabarra et al., 2015). Cultural control has also been shown to be effective in a number of cropping situations (Cormier et al., 2015) and now forms a large part of the advice given to British growers suffering infestations (ADHB, 2015). Despite these advancements control of this pest is far

from straight forward: chemical solutions make pesticide residue management, so critical on fruit crops, difficult (Diepenbrock et al., 2016) and cultural controls cost many man-hours, a worry for British growers in such an uncertain labour market (Miller, 2016). A highly efficacious, environmentally benign, IPM compatible control solution is still widely sought.

To date the microbiology of *D. suzukii* has been studied with the overarching theme of improving control, very few studies have explored the relationship of this species to its pathogens with the aim of understanding host-parasite coevolution. With *D. suzukii* being so closely related to the eminent model species *D. melanogaster*, it is potentially useful in comparative studies seeking to understand the divergence of immune system function and the ecoimmunological interaction of invasive species with native congeners.

Overview of the thesis

In chapter two we describe 18 new RNA viruses of D. suzukii from wild flies and larvae. We use a metatransciptomic approach to identify viruses infecting this fly in both its native (Japanese) and invasive (British and French) ranges. We describe eighteen new RNA viruses, including members of the Picornavirales, Mononegavirales, Bunyavirales, Chuviruses, Nodaviridae, Tombusviridae, Reoviridae, and Nidovirales, and discuss their phylogenetic relationships with previously known viruses using the conserved RdRp coding region of the viral genomes. We established the presence of these viruses in cDNA generated from pooled RNA extractions using RT-PCR and use RT-negative PCRs to confirm that these viruses are not present as endogenised viral elements (EVEs). The genomic structure of viruses including the location of key

conserved protein coding domains. We also detect 18 previously described viruses of other Drosophila species that appear to be associated with *D. suzukii* in the wild.

Chapter 3 describes the prevalence and host range of a subset of the viruses described in chapter two along with a number of viruses first described from native drosophila species. We used PCR to survey infection prevalence in five species of Drosophila: *D. melanogaster*, *D. obscura*, *D. subobscura*, *D. immigrans* and the invasive *D. suzukii*. D. subobscura showed the highest overall infection prevalence with 77.0% of flies infected with at least one virus. We find that *D. suzukii* has the highest diversity of viral infections with Teise virus to occurring at a high prevalence in this species. Infection by any virus was higher in native, Japanese *D. suzukii* than in invasive British populations. This finding was in line with the enemy release hypothesis (Keane and Crawley, 2002) although our data lack the temporal replication in native ranges needed to confirm trends in virus escape.

Chapter 4 describes the transcriptional response of two closely related species of *Drosophila*, *D. melanogaster* and *D. suzukii*, and shows how genome-wide expression varies under challenge by two different viruses. We found the genome-wide responses to an RNA virus (DCV) and a DNA virus (KV) to be well correlated in *D. melanogaster*. The correlation of response to these two virus types was less well correlated in females than in males highlighting sexual dimorphism in the response to this virus. We found that the anti-viral expression patterns of the two species in our study were less well correlated especially in males.

Future directions

In chapter two I suggest the most suitable possible candidate for investigation as a biological control agent to be the reovirus Eccles virus. The primary reason for this suggestion is its close phylogenetic relationship to a group of viruses previously advocated for the control of insect pests: the cypoviruses (Peng et al., 1998, Peng et al., 2000, Zeddam et al., 2003a). Crude isolation procedures of a pine moth cypovirus, *Dendrolimus punctatus* cytoplasmic polyhedrosis virus (DpCPV), were possible through serial passage in a substitutive host: *Spodoptera exigua* (Xiao et al., 2010). These techniques could be mirrored for Eccles virus. Immune compromised *D. melanogaster* such as Dicer$^{-2L811fsX}$ mutants could act as a suitable substitutive host and the resultant crude virus solution taken on to gradient centrifugation protocols as recently used in the isolation of KV virus (Palmer et al., 2018b). With a clean isolate of this virus, pathology studies could be undertaken to determine its lethality and therefore its part of its suitability as a biopesticide. In preliminary experiments conducted for this thesis we attempted to elicit infection in immune compromised *D. melanogaster* by injection with wild fly homogenate filtered through a 0.22µm filter. This included flies shown by PCR (chapter 3) to be infected with Eccles virus. Possibly due to low levels of virus in initial homogenate, no viruses of interest were redetected in these immune compromised lines. In attempts to increase viral titre serial passages were conducted, injecting homogenate from lab flies cyclically until mortality was observed. All lines were also injected with sterile buffer and passaged in the same way to act as a control against contamination. None of these experiments yielded significant mortality effects of viral extract over passaged control treatments and re-

examination of flies showing mortality revealed high levels of contamination with DCV, assumed to be contracted from the lab environment.

Levels of pathogenicity are not the only factor determining the suitability of a virus to biological control applications. One major obstacle to producing a reliable virus-based control product is the stability of virions outside of their host (Hunter-Fujita et al., 1998a). This is likely the reason why to date occulated Baculoviruses have predominated as the primary infectious agents in viral biopesticides. Baculoviruses are protected from the environment by a polyhedrin coat, occlusion body, that prevents desiccation and UV damage and extends the time virions remain infectious in a crop situation (Hunter-Fujita et al., 1998a). Most RNA viruses lack this occlusion body, including those discovered in this study but it may be possible to artificially provide a protective coat to viruses using modern encapsulation techniques. Although encapsulation of biological control agents has mainly been tested with entomopathogenic nematodes (Vemmer and Patel, 2013) some authors have suggested methods suitable for the encapsulation of virus particles (Inra, 1993). In a recent study Al-Handawi et al. (2018) were able to incorporate virions of cowpea mosaic virus (CPV) into a mineral calcite matrix. The structure of virions was protected from extreme chemical and thermal treatments, however, viable infectious particles were not recovered from the matrix after incorporation. This remains a fledgling technology and the artificial occlusion of virus particles improving their application as pest control agents is unlikely in the short to medium term.

Although in this project we have not been successful in isolating a viral biological agent lethal or stable enough to be taken forward directly to development we have made the first description of the viral diversity in *D. suzukii*, essential information in the continued hunt for viral biopesticide. It is possible that particularly pathogenic viruses are very rare in wild populations, although our understanding of the distribution of viruses is based largely around the study of viruses causing some form of disease, less is known about the distribution of non-pathogenic viruses. If the search for a viral biological control agent for *D. suzukii* is continued, then very large numbers of flies must be trapped from wild populations in order to grant the power needed to detect these rare viruses.

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Appendices

A

Table A.1 Known Drosophila Viruses detected in D. suzukii pools.

S1. Known *Drosophila* Viruses detected in *D. suzukii* pools. Viruses of *Drosophila* described by previous authors that were detected in our wild *D. suzukii* pools.

Name	Accession	Original Host	Taxon	Genome	Sample(s)	Ref.
Galbut	KP714099.1/KP714100.1	Dmel	Partitiviridae	dsRNA	UK2014, UK2015, UK2016, Japan2016	Webster et al. 2015
Chaq virus	KP714088.1	Dmel	Unclassified	-ssRNA?	UK2014, UK2015, UK2015	Webster et al. 2015
Dimm sigma virus	JF311401.1	Dimm	Rhabdoviridae	-ssRNA	France2013, UK2014, UK2016, Japan2016	Longdon et al. 2015
Corseley virus	KU754520.1	Dsub	Tombus-noda	+ssRNA	UK2014, UK2015	Webster et al. 2016
La Jolla virus	KP714073.1	Dmel	Iflaviridae	+ssRNA	France2013, UK2014, UK2015, UK2016	Webster et al. 2015
Muthill virus	KU754517.1	Dimm	Virgavirus	+ssRNA	UK2014, UK2015, Japan2016	Webster et al. 2016
Brandeis virus	MF953177	Dmel/Dsuz	Virgavirus	+ssRNA	UK2014, UK2016, France2013	Webster et al. 2016
Kinkell virus	KU754510.1	Dsus	Iflavirus	+ssRNA	UK2014, UK2015, UK2016	Webster et al. 2016
Dimm Nora virus	KF242511.1	Dimm	Noravirus	+ssRNA	UK2014, UK2015, UK2016, Japan2016	van Mierlo et al. 2014
Dmel Nora virus	JX220408.1, NC_007919.3, KP970094.1, KP970098.1, KP970100.1, KP970105.1	Dmel	Noravirus	+ssRNA	France 2013	Habayeb et al. 2006
Thikka virus	KP714072.1	Dmel	Picornavirales	+ssRNA	France2013, UK2015, UK2016, Japan2016	Webster et al. 2015
Prestney Burn virus	KU754507.1	Dsub	Sobemovirus	+ssRNA	UK2014, UK2015, UK2016, Japan2016	Webster et al. 2016
Buckhurst virus	KU754516.1	Dobs	Negevirus	+ssRNA	UK2015, UK2016, Japan2016	Webster et al. 2016
Drosophila A virus	NC_012958.1	Dmel	Picornavirales	+ssRNA	France2013, UK2014, UK2016, Japan2016	Brun & Plus 1980
Motts Mill virus	KP714076.1,KP714077.1	Dmel	Sobemovirus	+ssRNA	UK2015, UK2016, Japan2016	Webster et al. 2015
Craigies Hill virus	KP714085.1, KP714084.1	Dmel	Nodavirus	+ssRNA	Japan2016	Webster et al. 2015
Dkikkawai virus	SRR346732	Diptera	Fisavirus	+ssRNA	Japan2016	Webster et al. 2015
Bloomfield virus	SRR2063773	Dmel	Reoviridae	dsRNA	UK2014, UK2015, UK2016	Webster et al. 2015

B

Table B.1 Primers used for virus surveys

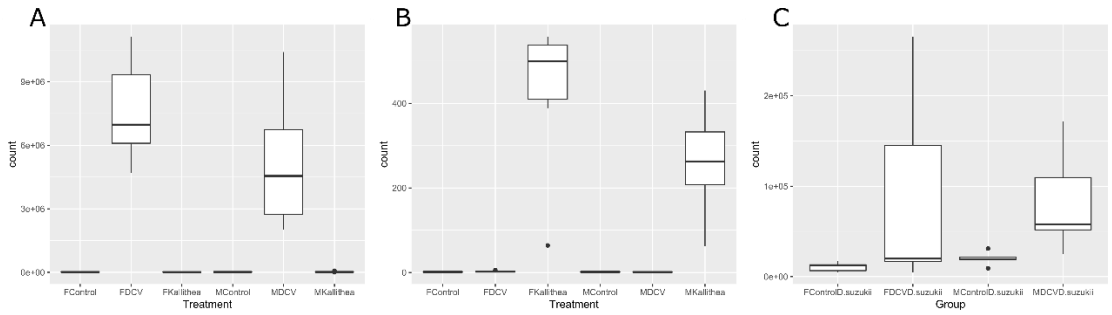
S2_Table. PCR Primer pairs for all newly discovered viruses

Primers used to detect new viruses by PCR. Melting temperature (T_m) as calculated by Primer3 plugin for Geneious 8.0.2. Annealing temperatures for used for reactions were universally 5°C lower than the stated melting temperature.

Virus	Primer name	Oligo	Product length	Tm
Teise Virus	Dsuz_Teise_seg1_00562F	GCTATGGTTCCGGGCTCAAG	713	60
	Dsuz_Teise_seg1_01274R	TCCTTTCCGCCGACATCAAC	713	60
	Dsuz_Teise_seg2_00570F	TGATCTTGGTCCCGGAAAGC	636	60
	Dsuz_Teise_seg2_01205R	GCGTCCATGCGTCCAAATAC	636	60
	Motts_like_bridgeB_02798F	CACTTGCCAGAAACCTTCCG	729	59.4
	Motts_like_bridgeB_00584R	CTCCAGTCACGACAACACAC	729	58.8
Medway Virus	Dsuz_Medway_01065F	AACGTCGGACTTCATCGAGC	502	60
	Dsuz_Medway_01567R	TCCAACATGTACGGCAAGCC	502	59.5
Ditton	Ditton_pol_03095F	TGATTGCAGGATTGGATGG	713	55.2
	Ditton_pol_03807R	CAGCATATCGGTTACAGAGC	713	56.2
Fuefuki virus	Dsuz_nido-like_03979F	ATACATGGAATCTGTGGAGGC	686	57.5
	Dsuz_nido-like_04665R	AGCATAATTGACAGTCGAGC	686	55.9
Snodland virus	Dsuz_Snodland_01372R	AGCACACTTGGTTCGTTGC	741	59.5
	Dsuz_Snodland_00632F	TCGGTTCACAGCCAGTATCC	741	59.3
Larkfield virus	Larkfield_02095F	TCAATCAAGCGAGTATCCACC	749	57.8
	Larkfield_02843R	ACGATGTTCAATGTCCTAGGC	749	58.1
Tama virus	Dsuz_Tama_C_01710R	AGGTTGATGGGATCGGATGG	645	59.2
	Dsuz_Tama_C_1066F	CATATGTTGACACTGGCTGCG	645	59.9
Mogami virus	Dsuz_Mogami_02095F	CCGTGGCGATATGTACTTGC	620	59.1
	Dsuz_Mogami_02714R	TCGGACACTGTAGACTGAGC	620	58.8
Dsuz Nora virus	Dsuz_Nora_Virus_02975F	ACCACCTGAGAACCTATGGC	804	59.1
	Dsuz_Nora_Virus_03778R	AAGTCGAGTCCTCTACCAACC	804	58.8
Barming virus	Dsuz_Barming_04850F	GTTGCCAATAAGCCTCCATTCC	686	59.9
	Dsuz_Barming_05536R	CCTGTTCTACTGCAGCTTGG	686	58.6
Cyril virus	Dsuz_Cyril_B_01230R	TGCTAGTTACTGGGTCACGC	918	59.8
	Dsuz_Cyril_B_313F	ATCCTCAGTTGGCTCGTCG	918	59.1
	Dsuz_Cyril_A_00954R	ATTTGCCGCTCTCAACATCG	649	59.3
	Dsuz_Cyril_A_00306F	GCTTCACATCCTCAGTTGGC	649	59.2
Naganuma virus	Dsuz_Naganuma_Virus_00173F	ATTCCAAGCCGAGACGACC	894	59.7
	Dsuz_Naganuma_Virus_01066R	TCGTTGGAGTCACATCCACC	894	59.8
Beult Virus	Dsuz_Beult_05955F	CAGTTGAACGTGTCTCTTAGGTG	836	59.3
	Dsuz_Beult_06790R	CCATCCGTGTATCCATTGGC	836	58.8
Saiwaicho virus	Dsuz_Saiwaicho_virus_B_03698R	TCTTGCACTTACCGACGAAG	995	59.7
	Dsuz_Saiwaicho_virus_B_02703F	CTGCTGAGAACCGACGATTC	995	58.7
	Dsuz_Saiwaicho_virus_A_03300R	TATCTTCTTGACCGACCGCC	678	59.4
	Dsuz_Saiwaicho_virus_A_02622F	GTGCTTCAACGTACGATCCG	678	59.4
Luckshill	Dsuz_Broad_bean_00442F	AGATGTCTAGTGCCGTGCC	867	59.5
	Dsuz_Broad_bean_01308R	TCAGCATGTCCAGTTGTTGC	867	59
Eccles virus	Eccles_virus_seg4_02305F	TACTATGCTACTCGTCGCGC	745	58.9
	Eccles_virus_seg4_03050R	CAATTGTTAGCTCCACCCGG	745	59.8
Kiln Barn virus	Dsuz_Kiln_Barn_03045F	TGTCGATCCAACCTCTCAACC	671	59.7
	Dsuz_Kiln_Barn_03715R	CGCTGCCATATTCCGTATAGC	671	59.1
Notori_virus	Dsuz_Notori_02969F	CAGAAGGAGACAACTTACGG	790	55.5
	Dsuz_Notori_03758R	GGTAATCTTGAAGGCCATCC	790	55.9
Brandeis virus	Dsuz_Murles_01176F	ATACGCGTGAATTGCAGTCG	782	59.4
	Dsuz_Murles_01958R	GAGTTCGCAGCATGAAGACG	782	59.6

C

Fig. C.1. Normalised counts mapping to virus genomes. A. counts of reads mapping to DCV from Dmel treatments, B. counts of reads mapping to KV PolB gene from Dmel treatments, C. counts of reads mapping to DCV from Dsuz treatments.



Papers arising from this thesis